

HUMAN γ -TRACE

PRIMARY STRUCTURE
LOCALIZATION TO NEUROENDOCRINE CELLS
AND
CONCENTRATION IN BODY FLUIDS

Helge Löfberg



MALMÖ 1982

Lund 1982



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av

HELGE LÖFBERG

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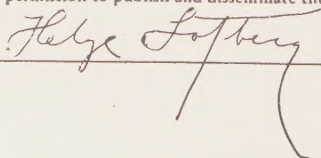
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Title and subtitle Human γ -trace: Primary structure, localization to neuroendocrine cells and concentration in body fluids.			
Abstract γ-Trace is a low molecular weight human protein with unknown function, occurring in body fluids. This thesis deals with the determination of the complete amino acid sequence of human γ-trace, its localization to neuroendocrine cells and concentration in human body fluids at various ages. γ-Trace was isolated from human urine, and antisera specific for γ-trace were raised in rabbits. An enzyme immunoassay, sensitive down to 30 μg γ-trace/l, and an enzyme amplified single radial immunodiffusion, sensitive down to 0.3 mg γ-trace/l, were devised for quantitation of γ-trace. The concentration of γ-trace in normal cerebrospinal fluid (CSF) and plasma was found to vary with age. The γ-trace level in CSF and plasma is high in infants and in the aged. In healthy adults the concentration of γ-trace (mean $\pm$ SD) in CSF is 5.4 ± 1.6 mg/l, in plasma 1.1 ± 0.34 mg/l, in mixed saliva 1.8 ± 0.88 mg/l and in urine 0.095 ± 0.057 mg/l. Immunohistochemical investigations of normal and neoplastic tissue from the monkey and man using the PAP-technique showed that γ-trace is localized to cells belonging to the peptidergic GEP neuroendocrine system in the cerebral cortex, adenohypophysis, pancreatic islets and adrenal medulla. The amino acid sequence of human γ-trace was determined by automated Edman degradations of the carboxymethylated polypeptide chain and fragments obtained by cyanogen bromide treatment and tryptic digestion after blocking of the lysine residues. The single polypeptide chain of γ-trace contains 120 residues. The proline residue at position 3 is partly hydroxylated. The calculated molecular weight of γ-trace is 13,260.			
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PRIMARY STRUCTURE, LOCALIZATION TO NEUROENDOCRINE

CELLS AND CONCENTRATION IN BODY FLUIDS

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MALMÖ 1982

The present thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

- I. Löfberg, H. & Grubb, A.O. (1979): Quantitation of γ -trace in human biological fluids; indications for production in the central nervous system. *Scand. J. Clin. Lab. Invest.* 39, 619-626.
- II. Löfberg, H., Grubb, A.O., Sveger, T. & Olsson, J.-E. (1980): The cerebrospinal fluid and plasma concentrations of γ -trace and β_2 -microglobulin at various ages and in neurological disorders. *J. Neurol.* 223, 159-170.
- III. Löfberg, H., Grubb, A.O. & Brun, A. (1981): Human brain cortical neurons contain γ -trace. Rapid isolation, immunohistochemical and physicochemical characterization of human γ -trace. *Biomed. Res.* 2, 298-306.
- IV. Löfberg, H., Strömblad, L.-G., Grubb, A.O. & Olsson, S.-O. (1981): Demonstration of γ -trace in normal and neoplastic endocrine A-cells of the pancreatic islets. An immunohistochemical study in monkey, rat and man. *Biomed. Res.* 2, 527-535.
- V. Löfberg, H., Nilsson, K.E., Strömblad, L.-G., Lasson, Å. & Olsson, S.-O. (1981): Demonstration of γ -trace in normal endocrine cells of the adrenal medulla and in pheochromocytoma. An immunohistochemical study in monkey, dog and man. *Acta Endocrinol. (Kbh)*. Accepted.
- VI. Grubb, A. & Löfberg, H. (1981): Human γ -trace, a basic micro-protein: Its amino acid sequence and presence in the adenohipophysis. Submitted.



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<u>Abbreviations:</u>	APUD,	amine precursor uptake and decarboxylation
	C1q,	the first subcomponent of complement
	CNS,	central nervous system
	CSF,	cerebrospinal fluid
	DEAE,	diethylaminoethyl
	DEPC,	diethylpyrocarbonate
	EIA,	enzyme immunoassay
	GEP,	gastro-entero-pancreatic
	PAP,	soluble complexes of horseradish peroxidase/ /rabbit anti-horseradish peroxidase
	SD,	standard deviation
	SDS,	sodium dodecyl sulphate

INTRODUCTION

In 1960 immunoelectrophoresis of human cerebrospinal fluid (CSF), using rabbit antisera against human CSF absorbed with human serum, revealed a slowly moving γ -globulin (9). When human CSF was concentrated about hundred-fold and analyzed with electrophoresis in agar gel, a narrow band containing the same protein was found in a position closely cathodally to the 7 S γ -globulins (33). Subsequently, several additional reports were published from different laboratories on this protein, and it was alternatively called " γ c globulin" (40), " γ CSF" (10), "post- γ protein" (7), " γ -trace" (25), " δ aT" (34) and "post- γ -globulin" (43). Today, the protein is called γ -trace, which is the name used in this work, or post- γ globulin.

γ -Trace has been demonstrated not only in concentrated CSF (10, 25, 33, 37, 42, 43), but also in urine from patients with proteinuria (7), in concentrated normal urine (70), ascitic and pleural fluids (25), colostrum (26), mixed saliva (8) and seminal fluid (11). At first, γ -trace was thought not to occur in blood plasma, but after fractionation and concentration of plasma, it was demonstrated also in this body fluid (8, 25). γ -Trace from different sources has been found to be immunochemically identical (8).

The biological function of γ -trace is still unknown. No cross-reactivity was demonstrated between γ -trace and IgG, IgA, IgM, κ or λ chains (37). With the aid of ion exchange chromatography, gel filtration and preparative electrophoresis γ -trace has been isolated from human CSF (26, 37, 40) and from urine from patients with tubular dysfunction (43, 71). The maximal final yield was 30 - 35 % (8). γ -Trace was found to be unstable and easily cleaved by such proteolytic enzymes as pepsin and papain (27). A shift of the electrophoretic pattern from a cathodal to a more anodal position was observed after degradation (8, 41, 68, 70).

Antisera against γ -trace were raised by immunizing rabbits with the isolated material. The concentration of γ -trace in CSF was determined with quantitative precipitin techniques using concentrated CSF. In three reports the mean concentration of γ -trace in CSF from normal adults was 9.7 (41), 8.3 (52) and about 2 (32) mg/l. In CSF from some patients with multiple sclerosis the values were lower. In normal infants and in infants with hydrocephalus the concentration of γ -trace in CSF was twice that in adults. No method for measuring the concentration of γ -trace in plasma was available. The high level of γ -trace in CSF could not be explained by a passive filtration of the protein from plasma (17). The concentration of γ -trace in CSF was not correlated with the total protein concentration in CSF and thereby indicated that γ -trace found in CSF was derived from the central nervous system (CNS) (38).

In attempts to identify the site of production of γ -trace tissues from Rhesus monkeys were cultivated and the incorporation of ^{14}C -lysine and isoleucine in γ -trace was studied with immunoelectrophoresis and autoradiography (24, 53). In vitro synthesis of γ -trace was found in tissues from various organs, but not in brain. It was abundant in the submaxillary gland, parotid gland, mammary gland, thyroid and testis. Immunoelectrophoresis revealed γ -trace to occur in tissue extracts from submaxillary gland in the monkey (24) and from human cerebral tissue (34).

The molecular weight of γ -trace as determined by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis has been given as 11 - 12,000 (68) and 11,500 (8), and 10,000 and 10,700 when measured with the ultracentrifugal technique (37). The sedimentation coefficient has been given as 1.5 S (37), 1.55 S (8), 1.6 S (24) and 2.6 S (43). No carbohydrate has been found in γ -trace (8). The isoelectric point of γ -trace is reported to be 9.0 (8), 9.3 and 8.0 (47) and 9.5 (35).

The amino acid composition of γ -trace has sometimes been found to include hydroxyproline (8, 43) and hydroxylysine (43), sometimes not (67, 68). The amino acid sequence of the 52 amino-terminal residues of γ -trace has recently been reported (67). However, 8 residues in the fragment were not identified. The amino-terminal residues of the degraded forms of γ -trace with anodal electrophoretic migration were recognized as numbers 5, 8 and 9 in the amino acid sequence of the native γ -trace molecule (67, 68).

AIMS OF THE INVESTIGATION

The purpose of this thesis was to try to fill in some of the gaps in our knowledge of human γ -trace. The specific aims of the investigation were to:

- A) Isolate γ -trace from human urine;
- B) Produce an anti- γ -trace antiserum;
- C) Develop a sensitive method for quantitation of γ -trace in human body fluids;
- D) Identify γ -trace-containing cells in various tissues from monkey and man;
- E) Determine the complete amino acid sequence of γ -trace isolated from human urine.

INVESTIGATIONS

A. ISOLATION OF γ -TRACE

The background information and details of the procedures used and results obtained are given in two of the papers of this thesis (I, III).

Material and Methods

Fresh urine containing non-degraded γ -trace was collected from uremic patients with advanced low molecular weight proteinuria. Proteolytic digestion of the urinary proteins was inhibited by addition of benzamidine hydrochloride and bacterial growth by addition of sodium azide. Most proteins of high molecular weight were removed from the urine by pressure ultrafiltration. The urine was concentrated, and alkaline protein fractions containing γ -trace were obtained in the void volume on DEAE-Sephadex ion exchange chromatography at a pH above 8.5 (I, III).

Results

The yield of the isolated non-degraded γ -trace was 13.2 %. On SDS-polyacrylamide gel electrophoresis and analytical agarose gel electrophoresis the isolated γ -trace produced only one band. No precipitates formed when the protein was tested with double radial immunodiffusion with the use of antisera against κ or λ or against IgG Fab or Fc fragments or on immunoelectrophoresis with use of a polyvalent antiserum against human plasma (I, III).

Discussion

In severe uremia the urinary excretion of low molecular weight proteins exceeds that in renal proximal tubular disease (29). This may be due to an increased plasma concentration of proteins with a molecular weight below 45,000 in patients with reduced glomerular filtration rate, resulting in a load exceeding the maximal tubular reabsorption capacity. A uremic inhibition of proximal tubular function or a damage to the tubuli co-existing with the damage of the glomeruli may also contribute to the advanced microproteinuria.

In some experiments several fractions obtained from the ion exchange chromatography column contained large amounts of acidic forms of degraded γ -trace (III). These fragments were derived from urine collected from patients with an indwelling catheter and asymptomatic bacteriuria, and the urinary proteins were obviously digested by bacterial enzymes. DEAE-Sephadex originally used in the isolation of γ -trace was later abandoned and replaced by DEAE-Sephacel, as the beads of some batches of DEAE-Sephadex released carbohydrate.

B. PRODUCTION OF ANTI- γ -TRACE ANTISERUM

The production and characterization of the anti- γ -trace antisera have been described in the same two papers as those about the isolation of γ -trace (I, III).

The γ -trace isolated was recycled three times on a column of Sephadex G-50 Superfine, emulsified with complete Freund's Adjuvant and was used for immunization of three rabbits without being coupled to any carrier protein (I). When the precipitating antibodies of the rabbit antisera against γ -trace were investigated by classical and crossed immunoelectrophoresis with the use of isolated γ -trace and several dilutions of CSF and uremic human plasma, only one cathodal precipitating arc appeared (I). The precipitating titer according to Becker (1) was 0.19 mg γ -trace/ml antiserum (III).

C. DEVELOPMENT OF A SENSITIVE METHOD FOR QUANTITATION OF γ -TRACE IN HUMAN BODY FLUIDS

The details of the construction of an enzyme immunoassay for quantitation of γ -trace and the results obtained, when the concentration of γ -trace was measured in various body fluids, are reported and discussed in two papers of the thesis (I, II).

Methods

The γ -Trace Standard: Directly after lyophilization a portion of the isolated γ -trace was carefully weighed and dissolved in a known volume of buffer (I). The protein concentration was controlled with a Biuret-Folin assay (14). In order to get a secondary standard, as similar as possible to the material to be assayed, CSF from a healthy volunteer was calibrated against the primary standard and used as working standard. The secondary standard was divided into small aliquots and stored at -24°C .

Enzyme Immunoassay for γ -Trace: A competitive type of enzyme immunoassay (EIA) was devised (2, I). We used alkaline phosphatase as label and a secondary antibody for separating free from antibody-complexed enzyme-labeled γ -trace. The following controls were included in each run of the EIA:

The non-specific binding of labeled γ -trace was checked by omission of the antiserum against γ -trace. These samples were pooled and used as a blank.

The maximal binding of enzyme-labeled γ -trace was measured by omission of non-labeled γ -trace in the competitive EIA.

The total enzyme activity of all the added labeled γ -trace was determined by omission of the washing steps.

The inter-assay precision and the niveau of the values obtained were estimated by inclusion of samples containing known concentrations of γ -trace.

The samples of the working standard used for the dose response curves were measured in triplicate and the clinical samples in duplicate.

Characterization of EIA: The following examinations ensured that the results of the assay were reliable and representative of the true concentration of γ -trace in the biological samples:

Titration of the antiserum: An antiserum titration curve was constructed to determine the most suitable dilution, 1:6,000, of the antiserum against γ -trace (I).

Accuracy: The closeness to the real or true value as estimated from recovery experiments was between 85 and 105 %. The dose response curves of different body fluids were found to be parallel (I).

Precision: The reproducibility or the degree of agreement of duplicate measurements was determined. The standard deviation was 12 % of the mean concentration. The precision was impaired since the supernatant was decanted by hand, on separation of free from complex-bound labeled γ -trace (I).

Sensitivity: The minimal detectable amount of γ -trace corresponding to 2 SD of the maximal absorbance of the dose response curve was approximately 30 $\mu\text{g/l}$ (I).

Stability: The solution of labeled γ -trace could be used after one year's storage at 4°C (I).

Deterioration of γ -Trace: After a few days' storage at room temperature in the absence of preservative, or after frequently repeated freezing and thawing, the γ -trace molecules in the biological samples, as well as in the working standard used in the EIA, were degraded and their binding characteristics were altered.

Enzyme Amplified Single Radial Immunodiffusion: This assay was used for comparative purposes and for quantitation of β_2 -microglobulin (I, II). Horse-radish peroxidase labeled swine anti-rabbit IgG antibodies were added in a second layer of the single radial immunodiffusion and the precipitates were visualized with 3-amino-9-ethylcarbazole. The method was simple and required no technically advanced facilities. In order to avoid too low a total protein concentration in the samples, a diluent containing 10 % (v/v) non-immunized rabbit serum was used when γ -trace was quantitated in fractions with a low protein content.

Quantitation of β_2 -Microglobulin: β_2 -Microglobulin is of roughly the same molecular weight, 11,815 (3), as γ -trace, 13,260 (VI), and was used as a reference protein. The concentration of β_2 -microglobulin was determined in CSF and plasma from patients of various ages and with various disorders (II). A secondary standard consisting of uremic plasma was calibrated against a pure solution with known concentration of β_2 -microglobulin and was used in the assay.

Results

An EIA was developed for quantitation of γ -trace, using pure γ -trace as a primary standard (I). The assay was sensitive down to approximately 30 $\mu\text{g/l}$. Also an enzyme amplified single radial immunodiffusion method sensitive down

to 0.3 mg/l was devised for quantitation of γ -trace and β_2 -microglobulin (I, II).

The concentration of γ -trace and β_2 -microglobulin in CSF and plasma varied with age (Table 1) (II). The concentration of γ -trace (mean $\pm$ SD) in CSF from 35 healthy adults was 5.4 ± 1.6 mg/l and that in plasma 1.1 ± 0.34 mg/l, as measured with the EIA (II). In normal infants less than one month old the concentration of γ -trace in CSF was three times that in adults. The mean concentration of γ -trace in CSF in healthy adults was 5.2 times, and in healthy infants 6.2 - 7.1 times, the corresponding level in plasma.

The concentration of γ -trace in mixed saliva was 1.8 ± 0.88 mg/l and in urine from healthy individuals 0.095 ± 0.057 mg/l (I). Urine from patients with renal disorders contained up to 21 mg γ -trace /l urine (I). The concentration of γ -trace in plasma from three anephric patients maintained on hemodialysis was 6.4, 11.5 and 14.5 mg/l. Recent unpublished investigations have shown that the mean value of γ -trace in seminal fluid from 24 men without any obvious disorder of the genital tract, as measured with the assay for γ -trace, was 51.0 mg/l with a SD of 8.1 (39).

In patients with infectious disorders of the CNS the concentration of γ -trace and above all, the concentration of β_2 -microglobulin in the CSF were significantly increased (II). In a few patients with impairment of the flow and absorption of CSF the concentrations of the two proteins in CSF were raised (II).

Discussion

The formation, flow and absorption of CSF (21, 36, 46, 56, 58), the permeability of the blood-CSF barrier (18, 30) and the glomerular filtration rate (29, 73) influence the concentrations of small proteins in CSF and plasma.

Table 1. The concentrations and gradients of γ -trace and β_2 -microglobulin in age groups of the reference population

Age category	N	CSF		Plasma		γ -trace		CSF		Plasma		β_2 -microglobulin		β_2 -microglobulin		γ -trace gradient	
		γ -trace (mg/l)	mean $\pm$ SD	γ -trace (mg/l)	mean $\pm$ SD	CSF/plasma	gradient mean $\pm$ SD	β_2 -microglobulin (mg/l)	mean $\pm$ SD	β_2 -microglobulin (mg/l)	mean $\pm$ SD	CSF/plasma	gradient mean $\pm$ SD	β_2 -m. gradient mean $\pm$ SD			
Newborn																	
37–42 weeks of gestation	9	18.5 $\pm$ 2.3		3.4 $\pm$ 1.4			6.2 $\pm$ 1.7	1.9 $\pm$ 0.52		3.9 $\pm$ 1.7			0.53 $\pm$ 0.15			12.6 $\pm$ 2.1	
2–4 weeks	4	19.1 $\pm$ 3.8		2.6 $\pm$ 0.47			7.1 $\pm$ 0.50	2.2 $\pm$ 0.44		4.4 $\pm$ 2.4			0.60 $\pm$ 0.22			15.8 $\pm$ 5.2	
1–3 months	2	11.6 $\pm$ 0.95		2.6 $\pm$ 0.37			4.6 $\pm$ 1.0	1.9 $\pm$ 0.17		6.6 $\pm$ 2.3			0.33 $\pm$ 0.09			14.2 $\pm$ 0.61	
3–12 months	7	4.2 $\pm$ 1.5		1.6 $\pm$ 0.55			2.9 $\pm$ 1.6	0.65 $\pm$ 0.19		2.3 $\pm$ 0.69			0.30 $\pm$ 0.12			9.5 $\pm$ 2.9	
1–20 years	7	3.6 $\pm$ 0.97		1.1 $\pm$ 0.21			3.2 $\pm$ 1.1	0.60 $\pm$ 0.18		1.7 $\pm$ 0.64			0.37 $\pm$ 0.12			8.8 $\pm$ 2.2	
21–40 years	16	5.0 $\pm$ 0.99		0.90 $\pm$ 0.15			5.6 $\pm$ 1.2	0.88 $\pm$ 0.27		1.2 $\pm$ 0.23			0.74 $\pm$ 0.23			8.1 $\pm$ 2.5	
41–60 years	11	5.2 $\pm$ 1.9		1.0 $\pm$ 0.17			5.1 $\pm$ 1.7	1.0 $\pm$ 0.18		1.4 $\pm$ 0.23			0.73 $\pm$ 0.12			7.0 $\pm$ 1.9	
>60 years	8	6.6 $\pm$ 1.6		1.5 $\pm$ 0.42			4.7 $\pm$ 1.8	1.3 $\pm$ 0.30		2.0 $\pm$ 0.39			0.66 $\pm$ 0.23			7.2 $\pm$ 1.9	
All adults >21 years	35	5.4 $\pm$ 1.6		1.1 $\pm$ 0.34			5.2 $\pm$ 1.6	1.0 $\pm$ 0.30		1.5 $\pm$ 0.42			0.72 $\pm$ 0.21			7.5 $\pm$ 2.3	

Disorders affecting any of these protein influencing mechanisms will interfere with the normal turnover and the concentrations of the proteins. The normal concentrations of γ -trace, β_2 -microglobulin and other proteins in CSF vary with the age of the individual (66, 72). Most proteins produced outside the CNS are considered to be transported through the blood-CSF barrier by passive filtration-diffusion and the concentration gradients of the proteins between CSF and plasma are correlated to the hydrodynamic volume of the proteins (17). Such filtration is not energy-consuming and results in a CSF/plasma gradient below 1 for unbound proteins.

In previous reports (32, 41, 52) the mean concentration of γ -trace in CSF from normal adults was estimated to be 9.7, 8.3, and 2 mg/l. Owing to the poor sensitivity of the precipitin technique, only concentrated samples of CSF were measured in these reports. The mean concentration of γ -trace, 5.4 mg/l, in CSF from normal adults in the present investigation (II) was of the same order of magnitude as those on record.

The mean CSF/plasma gradient of γ -trace, 5.2, was far above that expected for a protein passively filtered from plasma and suggested a local production of γ -trace in CNS although other explanations are possible, e.g. active transport or complex-binding of γ -trace. High concentrations of γ -trace in CSF in infants had been reported earlier (52) and the high CSF/plasma gradient of γ -trace in comparison with that of the reference-protein β_2 -microglobulin (II) indicated a high intrathecal production of γ -trace in the first month of life. The unexpected high concentration of γ -trace in seminal fluid and in mixed saliva suggested multiple sites of production of γ -trace (39, I). The high concentration of γ -trace in plasma and urine from patients with uremia indicated that γ -trace is normally catabolized in the kidneys (I).

Unlike what has been found in previous investigations (41, 52), the concentration of γ -trace in CSF was not significantly decreased in patients with multiple sclerosis (II). Earlier studies had not given the

age of the reference population and of the disorder group. Differences in the mean age of the groups may influence the results obtained.

A raised concentration of γ -trace in CSF may be caused by impaired flow and absorption of CSF and was found in one patient with calcifications and in two patients with tumors in the CSF-spaces (II). In a man with a metastasizing glucagon-producing and γ -trace-containing pancreatic tumor, the concentration of γ -trace in plasma was normal, suggesting a predominating extra-pancreatic origin of the circulating γ -trace (IV). The raised concentration of β_2 -microglobulin in CSF from patients with infectious disorders of the CNS may be explained by an increased intrathecal production of β_2 -microglobulin (II).

D. IMMUNOHISTOCHEMICAL LOCALIZATION OF γ -TRACE

Background information and methodological details in our attempts to localize γ -trace by means of immunohistochemical procedures are all given in four of the papers of the present thesis (III, IV, V, VI).

Material and Methods

Tissues were studied for the presence of γ -trace with the unlabeled peroxidase/anti-peroxidase (PAP) technique (61). The most crucial points of this method are reviewed below.

Sampling of Biopsy Specimens: Quick and experienced handling of fresh biopsy specimens taken without diathermy immediately at operation was found to be of fundamental importance to avoid falsely negative results. To avoid cracks due to contraction in volume, the specimens were rapidly cut into small pieces, 3 mm across. Within a few minutes the specimens were then frozen in a mixture of propane and butane to the temperature of liquid nitrogen. This snap-freezing procedure caused the water to freeze in the tissue without formation of large tissue-disrupting ice crystals (48).

Fixation: γ -Trace is a water-soluble protein prone to degrade when stored unfrozen or unfixed. Freeze-drying followed by vapor-fixation with the cross-linking bifunctional fixative diethylpyrocarbonate (DEPC) was used to reduce the diffusion and depletion of the γ -trace of the tissue and to protect it against degradation (51). The fixative did not destroy the immunoreactivity of γ -trace, it did not prevent antibodies from penetrating to the antigenic sites in the sections and preserved the light microscopical structure of the tissue. DEPC is believed to react preferentially with the free ϵ -amino group

of the alkaline amino acid lysine and with the carboxylic groups of aspartic and glutamic acid by forming amide bonds (74). γ -Trace is a protein with a high isoelectric point and contains 7 residues of lysine (VI), which favoured the use of this fixative. Conventional immersion fixation with 4 % (w/v) cold buffered paraformaldehyde (12) or with Bouin's fluid (4) or intravascular perfusion with 4 % (w/v) paraformaldehyde did not result in satisfactory immunostaining of γ -trace (IV).

Mounting on Slides: Retention of the sections on glass-slides offered difficulties, especially when the immunohistochemical procedures required up to 20 washings with detergent-containing buffers. No sections were lost when they were mounted on slides with 0.15 - 0.25 % (w/v) "white UHU coll glue" in water and then air-dried at 37°C for one hour (IV). This glue contains no biological compound, is soluble in water and not poisonous.

Immunohistochemical Technique: The unlabeled PAP technique of Sternberger (Fig. 1) was used with hydrogen peroxide and diaminobenzidine tetrahydrochloride as enzyme substrate (19, 61). The antiserum against γ -trace was applied at dilutions varying between 1:300 - 1:2,400. In order to keep the slightly soluble γ -trace in situ in the fixed tissue, each washing was never allowed to exceed 30 sec. Dual PAP immunostaining, carried out in two sequences using brown diaminobenzidine and blue 4-chloro-1-naphtol as substrates, was used to reveal two peptides in cells in one and the same section (62). Peptide-containing cells were also studied in 2 μ m thick adjacent sections cut from paraplast-embedded specimens.

General Remarks on Specificity: When a tissue specimen investigated is stained with oxidized diaminobenzidine, the specificity of the staining must be proved. Several types of undesired staining not specific for γ -trace can

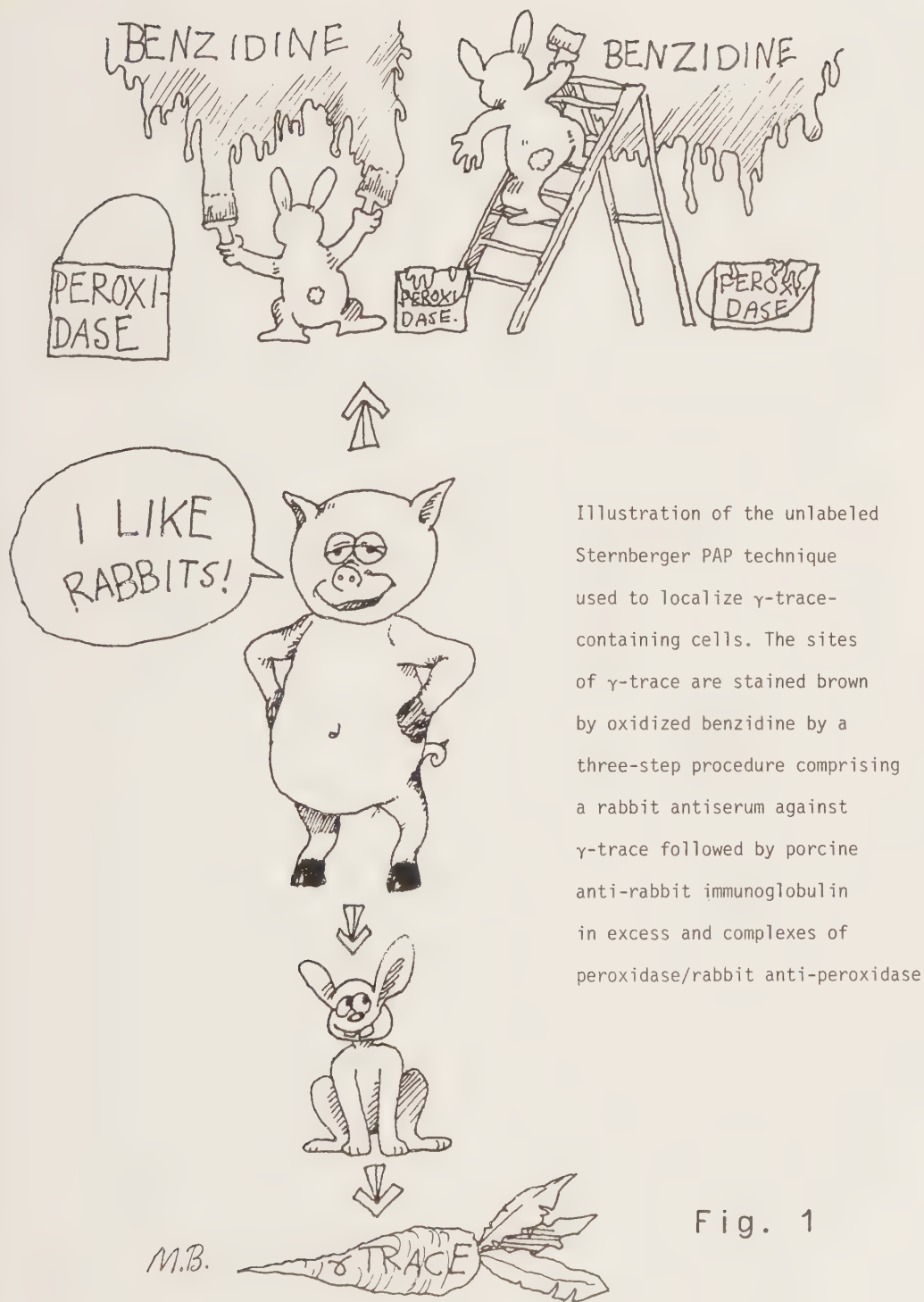


Illustration of the unlabeled Sternberger PAP technique used to localize γ -trace-containing cells. The sites of γ -trace are stained brown by oxidized benzidine by a three-step procedure comprising a rabbit antiserum against γ -trace followed by porcine anti-rabbit immunoglobulin in excess and complexes of peroxidase/rabbit anti-peroxidase

Fig. 1

be distinguished and three of them are discussed below.

1. Adhesion of the immunoglobulins of the antisera to the tissue specimen investigated owing to non-immunological binding interferes with the specificity of the method (65). This type of staining is independent of the specificity of the immunoglobulins and is seen especially in biopsy specimens rich in connective tissue.
2. The presence, in the antiserum, of any antibodies specific for compounds other than γ -trace can impair the specificity of the immunostaining. This specificity can be defined by characterization of the compounds that compete with the tissue antigen for the binding sites of the antibodies. Cross-reacting antibodies, reacting with structurally related antigens, may be detected when the immunoreactivity is absorbed by the cross-reacting compound, but the nature of this compound is often unknown (64, 69). The avidity of the cross-reacting antibodies is often low and the immunostaining is reduced when the antiserum is diluted. Natural heterophilic antibodies may exist in pre-immune serum and are not absorbed by γ -trace. Antibodies specific for impurities of the immunizing antigen may be detected after absorption of the antiserum with the pure antigen. The technique used to isolate the antigen utilized for this absorption must be different from that used when the immunogen was isolated as the absorbing antigen otherwise may contain the same impurities as the immunogen.
3. Endogenous peroxidase occurs in many tissues and its staining is independent of all the antisera in the assay.

Specificity of the Immunostaining of γ -Trace: The specificity of the immuno-reactivity of γ -trace was proved by using: a) the native antiserum, b) the absorbed antiserum, c) the complement subcomponent C1q depleted antiserum, d) sera from non-immunized rabbits, and e) immunosorbent isolated specific antibodies against γ -trace. The latter monospecific antibodies were obtained by affinity chromatography. This method might, however, remove the most avidly binding antibodies of high specificity (16).

A reduced, non-specific background staining, giving a high signal to noise ratio improved the efficiency of the method (54). The background staining disappeared when the total amount of the rabbit immunoglobulins in the solution used in the first step of the PAP technique was decreased, e.g. when only highly diluted antisera or isolated antibodies specific for γ -trace were used. The undesired staining was also reduced when hyperosmolar buffers containing bovine serum albumin were used (6, 22) and when the sections were protected from drying after incubation with the antisera. The penetration of the antibodies into the sections was improved by addition of a detergent, Triton X-100, to the buffers (20). When C1q-depleted antiserum or native antiserum against γ -trace was used, the immunostaining of γ -trace was identical, indicating that C1q-mediated non-specific binding (5) did not interfere with the results.

When the antiserum-specificity was investigated, no cross-reactivity was found between γ -trace and porcine glucagon, porcine cholecystokinin₃₉, synthetic gastrin₁₇, porcine insulin, human somatotropin, human prolactin, porcine corticotropin, synthetic corticotropin₁₋₂₈, bovine tyreotropin and human prealbumin. When various batches of γ -trace, isolated from three uremic patients, were used in the absorption experiments, the results were identical and suggested specificity for γ -trace. No natural antibodies could be detected.

The possibility of falsely positive immunostaining due to immunoreactivity to impurities of the immunized γ -trace could not be completely ruled out with certainty, as only one principal method of isolating γ -trace is known. The high immunoreactive endpoint, 1:2,400, of the dilutions of the antiserum weighed heavily against immunostaining not being specific for γ -trace.

Endogenous peroxidase occurred in erythrocytes and leukocytes in the tissue. The stain did not interfere with the interpretation of the immunostaining of γ -trace. The staining of endogenous peroxidase could be distinguished from the PAP immunostaining by a double staining procedure. First diaminobenzidine and hydrogen peroxide were added to the sections. This stained the sites of endogenous peroxidase brown and abolished the peroxidase activity. The immunostaining of γ -trace was then performed with 4-chloro-1-naphtol as enzyme substrate, staining the sites of the antigen blue (57). Inhibition of the activity of endogenous peroxidase by pretreatment of the sections with 0.3 % (v/v) hydrogen peroxide in methanol (63) was accompanied by loss of the immunoreactivity of γ -trace.

Precision: When the fixed and embedded biopsy specimens were repeatedly reinvestigated, the agreement between results was excellent. Examination of specimens, taken or fixed on separate occasions, revealed that the intensity of the immunostaining sometimes varied, indicating slight unintentional variation in the performance of the sampling and fixation.

Results

Immunohistochemical studies revealed immunoreactivity of γ -trace in neurons in the human cerebral cortex of the frontal and temporal lobes (III). The cytoplasm, the nuclei, and the dendrites were all immunoreactive. In pancreatic tissue from capuchin monkeys and Sprague-Dawley rats γ -trace was localized to the cytoplasm of the glucagon-producing A-cells of the islets of Langerhans. γ -Trace was also demonstrated in a human glucagon-producing A-cell tumor, a "glucagonoma" (IV). Immunoreactivity of γ -trace was found to be present in the cytoplasm of the chromaffin epithelial cells of the adrenal medulla in African green monkey, capuchin monkey, and the beagle and in the tumor cells of a human norepinephrine-producing pheochromocytoma (V). In simian and human adenohypophyses γ -trace was demonstrated in the cytoplasm of numerous small and large cells (VI).

Unpublished results of our recent investigation have also shown immunoreactivity of γ -trace in gastrin-containing endocrine cells in gastric mucosa from monkey and man (39). In prostatic glands from an adolescent monkey and from old men with nodular hyperplasia of the prostate, γ -trace has been found in the cytoplasm of the columnar cells lining the glands (39).

Discussion

In the interpretation of immunohistochemical results the limits of the method must be borne in mind. The PAP procedure visualizes antigens concentrated and stored inside the cell though antigens rapidly released from the cell may escape detection.

The present investigation showed that γ -trace may have something to do with neuroendocrine cells secreting regulatory peptides and hormones (31). With the possible exception of the prostatic cells, all the γ -trace-

containing cells belong to the paraneuronal peptidergic gastro-entero-pancreatic (GEP) neuroendocrine system (59) corresponding to the diffuse neuroendocrine system, based on the amine precursor uptake and decarboxylation (APUD) concept (49, 50).

In a previous report (24) in vitro incorporation of ^{14}C amino acids in γ -trace, produced by cultivated tissues from monkey, has shown that γ -trace is synthesized in the submaxillary gland, parotid gland, mammary gland, thyroid and testis but not in the brain. However, no detailed characterization of the nature of the cultivated γ -trace-producing cells was given and some cells, e.g. neurons, are difficult to keep alive in cultures.

The γ -trace-containing cells are now candidates for further studies concerning the production and physiological role of γ -trace. Our knowledge of the specificity of the antiserum against γ -trace acquired on immunoprecipitation in gels and in EIA is of limited value in immunohistochemical work. The sensitivity of double radial immunodiffusion and that of classical and crossed immunoelectrophoresis is lower than that of the PAP technique and cannot with certainty detect all undesired or non-precipitating antibodies. The specificity of EIA depends on the specific competition between labeled and unlabeled γ -trace for the antibodies. Therefore, it is the labeling and the purity of γ -trace and not the possible presence in the antiserum of antibodies against other compounds that decide the specificity of the competitive EIA technique (55). The lower dilution of the antiserum when used in the PAP method may also cause some differences in the specificity of the two assays.

E. THE COMPLETE PRIMARY STRUCTURE OF γ -TRACE

Background information on the molecular structure of γ -trace and the methodological details and the results of the determination of the amino acid sequence of γ -trace are given in two papers (III, VI) constituting the present thesis.

Methods

Pure carboxymethylated γ -trace was cleaved into 4 fragments by CNBr treatment (60) and, after citraconylation, into 9 fragments by tryptic digestion (13). One CNBr fragment was digested further by protease from Staphylococcus aureus V 8. The fragments were isolated by gel filtration and high voltage paper electrophoresis and the amino acid sequence of the γ -trace fragments was analyzed with the method of Edman & Begg (15) on a Beckman 890 C sequencer. The amino acid residues were identified by high performance liquid chromatography as well as by thin layer chromatography. The carboxy-terminal amino acids were identified after carboxypeptidase Y digestion (23). Almost all residues were identified twice in overlapping fragments.

Results

The complete amino acid sequence of all the 120 amino acid residues of human γ -trace was determined (Fig. 2) (VI). The calculated molecular weight was 13,260. The amino acid proline at position 3 of the single polypeptide chain of γ -trace was partly hydroxylated. Half-cystines were found at positions 73, 83, 97 and 117. The amino acid composition after acid hydrolysis agreed with that calculated from the sequence. When the amino acid sequence of γ -trace

¹ Ser-Ser-Pro-Gly-Lys-Pro-Pro-Arg-Leu-Val-Gly-Gly-Pro-Met-Asp-Ala-Ser-Val-Glu-Glu-²⁰
³⁰ Glu-Gly-Val-Arg-Arg-Ala-Leu-Asp-Phe-Ala-Val-Gly-Glu-Tyr-Asn-Lys-Ala-Ser-Asn-Asp-⁴⁰
⁵⁰ Met-Tyr-His-Ser-Arg-Ala-Leu-Gln-Val-Val-Arg-Ala-Arg-Lys-Gln-Ile-Val-Ala-Gly-Val-⁶⁰
⁷⁰ Asn-Tyr-Phe-Leu-Asp-Val-Glu-Leu-Gly-Arg-Thr-Thr-Cys-Thr-Lys-Thr-Gln-Pro-Asn-Leu-⁸⁰
⁹⁰ Asp-Asn-Cys-Pro-Phe-His-Asp-Gln-Pro-His-Leu-Lys-Arg-Lys-Ala-Phe-Cys-Ser-Phe-Gln-¹⁰⁰
¹¹⁰ Ile-Tyr-Ala-Val-Pro-Ser-Gln-Gly-Thr-Met-Thr-Leu-Ser-Lys-Ser-Thr-Cys-Gln-Asp-Ala¹²⁰

The complete amino acid sequence of human γ -trace. The single polypeptide chain contains 120 residues and has a molecular weight of 13,260. The amino acid proline at position 3 of the γ -trace chain is partly hydroxylated. Half-cystines are found at positions 73, 83, 97 and 117.

Fig. 2

was compared with those of the neuroendocrine peptides, similarities were found between glucagon, corticotropin and γ -trace. Eight of the 35 amino-terminal amino acids of γ -trace were in common with those of human corticotropin₃₉ and 7 with those of human glucagon₂₉ (VI).

Discussion

In a recent study (67) 44 of the 52 amino-terminal amino acid residues of γ -trace were identified and their sequence was found to agree with that presented here. The acidic degraded forms of γ -trace were observed to be devoid of 4, 7 or 8 amino acids of the amino-terminal end of the basic form of γ -trace (67, 68). Whether the basic γ -trace molecule isolated from urine and comprising 120 residues is the polypeptide chain of γ -trace demonstrated in neuroendocrine cells, or is a proteolytic product of a larger molecule, remains to be investigated. No cross-reactivity between human γ -trace and porcine glucagon and corticotropin was found with the immunohistochemical technique (IV, VI) despite the similarities found on comparison between the primary structures of the three polypeptide chains.

The amino acid composition of γ -trace has sometimes been reported to include hydroxyproline (8, 43) and hydroxylysine (43), sometimes not (67, 68). As mentioned above, the present study showed a hydroxylation degree of about 50 % of the proline residue at position 3 of the polypeptide chain when three preparations of pure γ -trace were investigated. Hydroxyproline was identified both in the uncleaved polypeptide chain and in the corresponding tryptic and cyanogen bromide fragments by amino acid analyses and high performance liquid chromatography of the corresponding phenylthiohydantoin derivatives. No hydroxylation of any other proline residue or any lysine residue could be found, however.

SUMMARY

γ -Trace was isolated from human urine, and antisera specific for γ -trace were raised in rabbits.

An enzyme immunoassay, sensitive down to 30 μg γ -trace/l, and an enzyme amplified single radial immunodiffusion, sensitive down to 0.3 mg γ -trace/l, were devised for quantitation of γ -trace. The concentration of γ -trace in normal cerebrospinal fluid (CSF) and plasma was found to vary with age. The γ -trace level in CSF and plasma is high in infants and in the aged. In healthy adults the concentration of γ -trace (mean $\pm$ SD) in CSF is 5.4 ± 1.6 mg/l, in plasma 1.1 ± 0.34 mg/l, in mixed saliva 1.8 ± 0.88 mg/l and in urine 0.095 ± 0.057 mg/l.

Immunohistochemical investigations of normal and neoplastic tissue from the monkey and man using the PAP technique showed that γ -trace is localized to cells belonging to the peptidergic gastro-entero-pancreatic (GEP) neuroendocrine system in the cerebral cortex, adenohypophysis, pancreatic islets and adrenal medulla.

The amino acid sequence of human γ -trace was determined by automated Edman degradations of the carboxymethylated polypeptide chain and fragments obtained by cyanogen bromide treatment and tryptic digestion after blocking of the lysine residues. The single polypeptide chain contains 120 residues and the sequence is S S P G K P P R L V G G P M D A S V E E E G V R R A L D F A V G E Y N K A S N D M Y H S R A L Q V V R A R K Q I V A G V N Y F L D V E L G R T T C T K T Q P N L D N C P F H D Q P H L K R K A F C S F Q I Y A V P S Q G T M T L S K S T C Q D A. The proline residue at position 3 is partly hydroxylated. The calculated molecular weight of γ -trace is 13,260.

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Quantitation of γ -trace in human biological fluids: indications for production in the central nervous system

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γ -Trace was purified in large amounts from urine and used for the production of a specific rabbit antiserum. An enzyme immunoassay for quantitation of γ -trace was developed using the pure protein as a primary standard. Its sensitivity was approximately 30 $\mu\text{g/l}$. An enzyme amplified single radial immunodiffusion was developed as well. Its sensitivity was approximately 0.3 mg/l. These assays allowed quantitation of γ -trace in normal human biological fluids. The following results were obtained (mean $\pm$ SD): cerebrospinal fluid: 5.8 ± 2.2 mg/l, plasma: 1.1 ± 0.42 mg/l, saliva: 1.8 ± 0.88 mg/l and urine: 0.095 ± 0.057 mg/l. Plasma samples from patients with advanced renal failure revealed γ -trace values up to 13 times the normal mean plasma value. The results indicate a production of γ -trace in the central nervous system and that the protein is primarily catabolized by the kidney.

Key-words: cerebrospinal fluid proteins; immunoenzyme techniques; proteinuria

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Abbreviations: CSF = cerebrospinal fluid; SDS = sodium dodecyl sulphate; PBS = 0.05 mol/l phosphate buffer, pH 7.4 with 0.1 mol/l NaCl; and BBS = 0.075 mol/l barbital buffer, pH 8.6 with 2 mmol/l MgCl_2 and 10% (v/v) non-immune goat serum.

An alkaline trace protein was demonstrated in urine from patients with tubular proteinuria and in normal cerebrospinal fluid (CSF) in 1961 [3, 5, 24]. The same protein was later shown to be present in normal human serum, urine, colostrum, saliva and semen as well as in ascitic fluid [4, 6, 11, 12]. The protein has been named γ -trace, post- γ -protein, post- γ -globulin, γ CSF,

δ a T and γ c globulin [3, 5, 11, 17, 18, 21, 22]. The function of the protein is unknown and controversy exists concerning its site of production [10, 17]. Its concentration in plasma, urine, CSF and other human body fluids is low and cannot therefore be determined by common immunochemical procedures based upon immunoprecipitation in gels [16, 23, 26]. A quantitative procedure sufficiently sensitive to allow determination of γ -trace in all these biological

fluids has not been described as yet. The purpose of this work was to construct such a method and determine the concentration of γ -trace in various normal human biological fluids.

MATERIAL AND METHODS

Human body fluids. Samples of plasma, urine and mixed saliva were collected from healthy laboratory personnel. Three plasma samples were taken from anefric patients maintained on haemodialysis.

CSF and plasma were taken from one healthy individual and from a selected group of patients with minor neurological complaints but with normal concentrations of total CSF proteins, normal CSF/plasma IgG- and albumin ratios and normal CSF electrophoretic protein patterns.

Antisera. Peroxidase conjugated swine immunoglobulins against rabbit IgG were obtained from DAKO immunoglobulins Ltd, Copenhagen, Denmark. All other antisera were available in our laboratory.

Miscellaneous chemicals. DEAE Sephadex A 50, Sephadex G 25, Sephadex G 50, Sephadex G 75 and Dextran T 10 were purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden, agarose from Marine Colloids Inc., Rockland, Maine, U.S.A., Coomassie Brilliant Blue R 250 from Schwarz/Mann, Orangeburg, N.Y., U.S.A., dimethylformamide from Mallinckrodt Inc., St Louis, Mo., U.S.A., 3-amino-9-ethylcarbazole and alkaline phosphatase type VII from calf intestine from Sigma Chemical Company, St Louis, Mo., U.S.A. Purified human albumin was from Behringwerke AG, Marburg, W. Germany and bovine albumin from Amour Pharmaceutical Company Ltd, Eastbourne, England. Sodium dodecyl sulphate, acrylamide and N,N'-methylene bisacrylamide, Folin & Ciocalteu's phenol reagent, diethanolamine, 4-nitrophenyl disodium orthophosphate, hydrogen peroxide 30% and glutaraldehyde solution 25% were obtained from British Drug Houses Ltd, Poole, England. Heparin 5,000 IE/ml was from Vitrum, Stockholm, Sweden and complete Freund's Adjuvant from Difco Laboratories, Detroit, Mich., U.S.A. All other

chemicals were of reagent grade and obtained from British Drug Houses, Ltd, Poole, England.

Isolation of γ -trace. A detailed report of the procedure will be given elsewhere [19]. Briefly, 30 l urine from a patient with tubular proteinuria were concentrated by pressure ultrafiltration and the protein fraction desalted by Sephadex G 25 column chromatography and finally, lyophilized. Acid and neutral proteins of the protein fraction were removed with the use of a DEAE-Sephadex A 50-column in 0.05 mol/l Tris-HCl buffer, pH 8.8 and γ -trace was finally freed of remaining contaminants by recycling the eluate from the ion-exchange column through a column of Sephadex G 50 Superfine. Pure γ -trace was stored at room temperature as a lyophilized salt-free powder.

Immunochemical and electrophoretic methods. Agarose gel electrophoresis was performed as described by Johansson [14], sodium dodecyl sulphate- (SDS) polyacrylamide gel electrophoresis as reported by Laemmli [15], double radial immunodiffusion according to Ouchterlony [25], classical immunoelectrophoresis as described by Scheidegger [27] and crossed immunoelectrophoresis as reported by Garrot [8].

Production of antiserum. 3 mg γ -trace was dissolved in 1.5 ml 0.15 mol/l NaCl and emulsified with 1.5 ml complete Freund's Adjuvant. The emulsion was injected in the back feet and at a few subcutaneous sites on the backs of three rabbits. Identical booster injections were given 3 weeks later and the rabbits were then bled every 2 weeks.

Enzyme amplified single radial immunodiffusion. The procedure of Mancini *et al.* [20] was used but the agarose was dissolved in 0.025 mol/l barbital buffer pH 8.6 containing 10% Dextran T 10 to augment the immunoprecipitation and 2% heparin-solution (5,000 IE/ml) to decrease non-specific protein binding to the agarose gel. The diffusion period was 36 h. To remove free unprecipitated proteins, the gel was washed in 0.15 mol/l NaCl for 30 min. The gel was then covered with a wet filter paper and 2 cm soft dry blotting paper, on the top of which was placed a weight. In 10 min most saline was absorbed from the gel. The washing procedure was

repeated twice. The gel was then incubated for 30 min in a moist chamber with peroxidase-labeled swine immunoglobulins against rabbit IgG, diluted 1:20 in PBS to which 3% non-immunized swine serum was added. The washing procedure was then performed three times. The precipitates of the gel were visualized by adding the substrate of the peroxidase, viz 20 mg 3-amino-9-ethylcarbazole dissolved in 2.5 ml dimethylformamide, 50 ml 0.05 mol/l acetate buffer, pH 5.0 and 25 μ l hydrogen peroxide 30%. The immunoprecipitates were stained wine-red within 10–15 min and the staining process was stopped by rinsing the gel in water. The gel was dried in front of a hot air fan. The amplifying technique was similar to the one used by Ingild [13].

The γ -trace standard. 1.00 mg γ -trace was dissolved in 1.00 ml 0.05 mol/l phosphate buffer pH 7.4 with 0.1 mol/l NaCl (PBS) directly after lyophilization. The protein concentration in the solution was in addition measured by the heated Biuret-Folin protein assay of Dorsey [7] which gives equal absorbance values for different proteins. This procedure gave a concentration value of 1.03 mg/ml for the primary γ -trace standard when pure human albumin was used as protein standard. The primary standard was stored at -70°C . A secondary γ -trace standard consisting of CSF from one healthy person was carefully calibrated against the primary standard and used as a working standard when the γ -trace concentration in biological fluids was determined. The secondary standard was divided into small aliquots and stored at -24°C .

Enzyme labeling of γ -trace. Ammonium sulphate solution containing alkaline phosphatase [2] was carefully dialyzed against PBS to completely remove the ammonium sulphate. Five microlitres of PBS containing 5% (w/v) glutaraldehyde [1] was added to 250 μ l PBS containing 500 μ g alkaline phosphatase and 150 μ g pure γ -trace. After overnight incubation the solution was fractionated on a column of Sephadex G-75 in PBS to remove unreacted glutaraldehyde and monomeric γ -trace from the enzyme-labeled γ -trace eluted in the void volume. Bovine albumin and sodium azide were added to the enzyme-labeled γ -trace solution to final concentrations of 40 g/l and 0.2 g/l, respectively, and the solution was stored at 4°C . After storage for

1 year this solution still worked without deterioration in the enzyme immunoassay described below and no precipitate formation in the solution could be observed.

Enzyme immunoassay. A sequential [28] saturation, competitive type of enzyme immunoassay was employed with the use of a secondary antibody to separate free and antibody-complexed enzyme-labeled γ -trace. The rabbit antiserum against γ -trace was diluted (1:6,000) with 0.075 mol/l barbital buffer, pH 8.6, containing 2 mmol/l MgCl_2 and 10% (v/v) non-immune goat serum (BBS) before use in the enzyme immunoassay. An antiserum titration curve showed that this dilution resulted in an antibody-binding of approximately 75% of the enzyme activity in the enzyme-labeled γ -trace solution used in the immunoassay.

A typical procedure was as follows. Fifty microlitres of a dilution (in BBS) of a standard, control or test sample was mixed with 100 μ l antiserum against γ -trace and the mixture was incubated for 4 h at room temperature. Fifty microlitres of the enzyme-labeled antigen solution (diluted 1:400 with BBS) was then added. After 8 h or overnight at room temperature 50 μ l non-immune rabbit serum (diluted 1:1000 with BBS) followed by 100 μ l of goat antiserum against rabbit IgG (diluted 1:10 with BBS) was added. After 3 h at room temperature 1 ml barbital buffer was added and the tube was centrifuged at 1300 *g* for 15 min at 4°C and the supernatant was cautiously decanted. The washing procedure was repeated once. 400 μ l of a solution of the enzyme substrate (1 mol/l diethanolamine buffer, pH 10.0, with 1.0 mmol/l MgCl_2 and 14 mmol/l 4-nitrophenyl disodium orthophosphate) was then added to the test tube which thereafter was vortex-mixed and incubated exactly 30 min in a 37°C waterbath. The reaction was stopped by addition of 2 ml 0.1 mol/l NaOH and the absorbance at 405 nm measured.

The standard samples were measured in triplicate and all test and control samples in duplicate. Suitable dilutions of normal CSF and plasma were found to be 1:60 and 1:15, respectively, whereas normal urine was measured undiluted.

In each immunoassay run the following controls were included:

Test tubes without any antiserum against

γ -trace to test non-specific binding of the enzyme-labeled γ -trace. These tubes were used as blanks in the spectrophotometric measurements.

One test tube without non-labeled γ -trace to check maximal binding of enzyme-labeled γ -trace.

One test tube containing only the enzyme-labeled γ -trace to test the added total enzyme activity.

Two test tubes with plasma samples of known γ -trace concentrations.

Urine creatinine. The procedure of Jaffé was used [9].

RESULTS

Purity of γ -trace used as primary standard and for immunization. The isolated protein gave only one band when analyzed by analytical agarose gel electrophoresis and by 15% SDS-polyacrylamide gel electrophoresis and produced no precipitate when used as antigen in immunoelectrophoresis with use of a polyvalent antiserum against human plasma. No precipitates were formed when the protein was tested in double radial immunodiffusion with use of antisera against κ - or λ -chains or against IgG Fab- or Fc-fragments. When injected into a rabbit the protein produced an antiserum which without any absorption was monospecific for γ -trace. The isolated protein had a unique amino-terminal amino acid sequence [19].

Antiserum specificity. When the unabsorbed antiserum against γ -trace was tested by classical and by crossed immunoelectrophoresis with use of pure γ -trace and several dilutions of CSF and of normal and uremic human plasma only one cathodal precipitation arc was obtained.

The enzyme immunoassay for γ -trace

Antiserum titration. Dilutions of enzyme-labeled γ -trace solution and antiserum against γ -trace which gave convenient absorbance values in the final spectrophotometric measurements of the enzyme immunoassay were determined by antiserum-titration experiments. An antiserum-titration curve is given in Fig. 1. The low antiserum dilutions produced precipitates with maximal enzyme activities amounting to 55% of

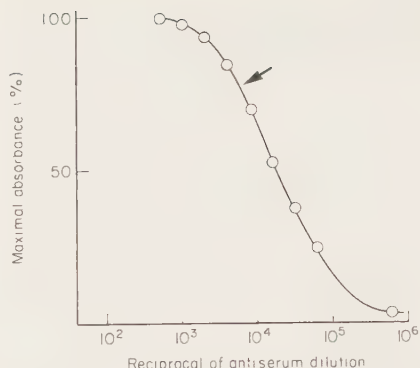


FIG. 1. Titration of rabbit antiserum in the presence of optimal concentration of γ -trace enzyme conjugate. Arrow indicates antiserum dilution used for assay.

total enzyme activity in the added enzyme-labeled γ -trace solution. When normal rabbit serum or unrelated rabbit antisera replaced the antiserum against γ -trace enzyme, activities of 1–2% of maximally precipitated activities were found. An antiserum dilution which precipitated 75% of the maximal precipitate activity was used in the assay.

Accuracy. The accuracy of the assay was tested by running dose-response curves for the primary γ -trace standard, normal human CSF and normal and uremic human plasma. As can be seen from Fig. 2 these curves were parallel. When a plasma sample was run through a column containing an anti- γ -trace immunosorbent the immunoassay could not detect any γ -trace in the eluted plasma. The recovery of the

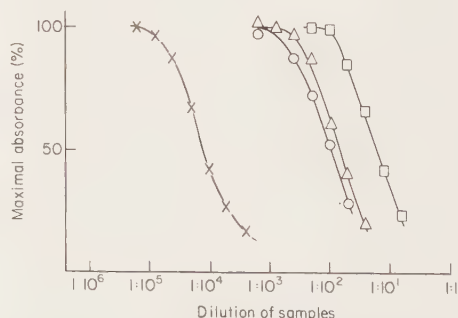


FIG. 2. Dose-response curves obtained with the primary standard (x), uremic plasma (o), CSF, the secondary standard (Δ) and normal plasma (□).

TABLE I. The γ -trace concentration in paired samples of plasma and CSF

Subjects	Quantitative method: Enzyme immunoassay		
	CSF conc (mg/l)	Plasma conc (mg/l)	CSF/plasma concentration ratio
30 paired samples	Mean: 5.8 Range: 3.2–12.5 SD: 2.2	Mean: 1.1 Range: 0.63–2.5 SD: 0.42	Mean: 5.5 Range: 2.3–14.6 SD: 2.5
One healthy volunteer	5.4	1.3	4.2

Subjects	Quantitative method: Amplified single radial immunodiffusion		
	CSF conc (mg/l)	Plasma conc (mg/l)	CSF/plasma concentration ratio
30 paired samples	Mean: 7.3 Range: 4.8–14.7 SD: 2.4	Mean: 1.4 Range: 0.79–2.5 SD: 0.43	Mean: 5.5 Range: 2.5–10.3 SD: 2.1
One healthy volunteer	6.2	1.2	5.2

assay was determined by adding 10 μ l of a solution containing 0.1 mg γ -trace/ml to 0.99 ml plasma. The γ -trace was quantitated and the calculated recovery was between 85 and 105%.

Precision. As calculated from fifty-seven duplicate determinations the standard deviation was found to be 12% of the mean for high-range and 10% for low-range concentrations.

Sensitivity. The sensitivity of the enzyme immunoassay was found to be approximately 30 μ g/l (2 SD of the maximal absorbance of the dose-response curve).

Stability. The solution of enzyme-labeled γ -trace was stored at +4°C, and gave similar or identical standard dose-response curves during a period of at least 1 year.

Enzyme amplified single radial immunodiffusion. This procedure was used mainly for comparative

purposes. The standard deviation of thirty duplicate determinations was 11% of the mean. The assay had a sensitivity of about 300 μ g/l and therefore could not be used for measurements of the γ -trace concentration in normal urine.

The γ -trace concentration in various biological fluids. Paired samples of plasma and CSF were collected from normal individuals and from patients with minor neurological complaints but with normal CSF total protein and CSF IgG. Their concentration of γ -trace was measured with the enzyme immunoassay and with the amplified single radial immunodiffusion. The results are shown in Table I. Both methods gave concentration values for CSF which were 5.5 times the plasma values.

When the γ -trace concentration was measured in plasma samples from healthy laboratory personnel the results were very similar to those obtained for the plasma samples of the paired CSF-plasma samples (Table II).

TABLE II. The γ -trace concentration in plasma, mixed saliva and urine from healthy individuals

Quantitative method	Plasma conc (mg/l)	Mixed saliva conc (mg/l)	Urine (mg/l)
Amplified single radial immuno-diffusion	n: 46 Mean: 1.3 Range: 0.72–1.7 SD: 0.26	n: 30 Mean: 1.8 Range: 0.36–4.8 SD: 0.88	
Enzyme immunoassay			n: 19 Mean: 0.095 (8.0 mg/mol creatinine) Range: 0.033–0.29 (5.2–13.3 mg/mol creatinine) SD: 0.057 (1.91 mg/mol creatinine)

The γ -trace concentration in mixed saliva samples from healthy individuals is given in Table II. The mean saliva concentration was somewhat higher than the mean plasma concentration. Although the difference was small it was statistically significant ($P < 0.01$ as tested with Student's t test for groups). Table II gives in addition the γ -trace concentration in urine from healthy individuals.

When plasma samples from three anefric patients maintained on haemodialysis were analyzed, the γ -trace concentrations were found to be: 6.4, 11.5 and 14.5 mg/l.

DISCUSSION

In the elaboration of the enzyme immunoassay two unexpected difficulties were encountered. Anticoagulants (citrate or EDTA) in some of the plasma samples complexed with so much of the Mg^{2+} ions in the original substrate solution that the alkaline phosphatase activity was inhibited. This problem was solved by using a higher Mg^{2+} concentration in the final substrate solution and Mg^{2+} -containing diluents for the plasma samples. The second difficulty was the endogenous alkaline phosphatase of the samples and the enzyme that contaminated the stabilizing protein. This background enzyme activity was reduced, when the two final washings of the immunoprecipitates were carried out with a non-protein buffer.

When the γ -trace concentration in plasma and CSF was measured by both the enzyme immunoassay and by the amplified single radial immunodiffusion the results of the two methods showed a good correlation ($r = 0.8$). However, minor, but statistically significant ($P < 0.01$ as tested with Student's t test), differences between the results were obtained (Table I). The reason for the differences is not known but may be related to possible differences in the molecular population of γ -trace in the standard and in the various samples.

The enzyme immunoassay described in this work has a sensitivity which allows quantitation of γ -trace in all tested biological fluids, has an acceptable precision and permits fifty test samples to be analyzed within 24 h by one technician. The enzyme-labeled γ -trace marker is stable and can be used for at least 1 year. This

procedure therefore seems suitable for the quantitation of γ -trace in both research and clinical routine work.

The enzyme amplified single radial immunodiffusion described in this paper has a sensitivity limit which does not allow determinations of γ -trace in all human biological fluids. Despite this it is technically simple and permits quantitation of the γ -trace concentration in CSF, saliva and plasma samples.

The results concerning the γ -trace concentration in various human body fluids give indications for the sites of production and catabolism of the protein. When the γ -trace concentration was determined in paired CSF and plasma samples the concentration in CSF was always much higher than the corresponding plasma concentration. A mean CSF/plasma concentration ratio of 5.5 was obtained by both the enzyme immunoassay and by the amplified single radial immunodiffusion. These results indicate a production of γ -trace in the central nervous system. Although this explanation for the high CSF/plasma concentration ratio is the most straight-forward other explanations may be offered. For instance, there may be an active transport of γ -trace from plasma into CSF. Although this mechanism has been suggested previously [12, 16] no evidence for it has been presented. Another possible explanation is that the molecular population of γ -trace in CSF differs from that in plasma and that the two populations therefore react in different stoichiometric ways with the antiserum. Even if this possibility has not been studied very extensively, available evidence [4] does not favor it.

The paired samples of plasma and CSF were collected from patients with minor neurological complaints and it may therefore be argued that the results are not valid for healthy individuals. Although this argument cannot be definitively rejected, several parameters tested argue against it. The patients had, for instance, normal CSF total protein, normal CSF/plasma ratios of IgG and albumin and normal CSF electrophoretic protein patterns. In addition, one healthy individual, who volunteered for the lumbar puncture, had a CSF and plasma concentration of γ -trace which were very close to the mean CSF and plasma values for this patient group. When the mean plasma concentration of γ -trace was determined in a group of healthy individuals the value did not differ statistically (as tested with

Student's t test) from the mean plasma value for the patient group.

The very high γ -trace concentration found in plasma samples from patients with advanced renal failure strongly indicates that γ -trace, like most plasma proteins of low molecular weight, are normally mainly catabolized in the renal tubular cells after filtration through the glomeruli.

The γ -trace concentration in normal urine was very low (about 1/13th of the normal plasma value). Much higher values (up to 21 mg/l) were obtained when a limited number of urine samples from patients with tubular disorders were analyzed. These findings agree with the hypothesis above concerning the catabolism of γ -trace.

An unexpected observation was that the γ -trace concentration in saliva was higher than that in plasma. The difference was comparatively small but statistically significant ($P < 0.01$ as tested with Student's t test). This result indicates a possible production of γ -trace in the parotid and/or submaxillary salivary glands and agrees with the results of Hochwald *et al.* who demonstrated a significant production of γ -trace in *in vitro* cultures of rhesus monkey submaxillary gland tissue [10].

Although no values for the γ -trace concentration in plasma, saliva and urine have been reported previously three publications concerning the γ -trace concentration in CSF have appeared. The mean CSF concentrations given by MacPherson (9.7 mg/l) and Pepe & Hochwald (8.3 mg/l) are close to the mean CSF concentration of this work [23, 26] while the value given by Laterre (2 mg/l) is considerably lower [16].

To summarize, the quantitative results of this work concerning the γ -trace concentration in various body fluids indicate a production of the protein in the central nervous system and probably some production in salivary glands. The protein probably passes the blood brain barrier mainly from CSF to the vascular space and is normally rapidly filtered through the glomeruli of the kidney and catabolized in the renal tubular cells.

Studies concerning whether quantitations of γ -trace in biological fluids can be used to detect and follow pathophysiological processes in the central nervous system, in the salivary glands and in the kidneys are under way.

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Original Investigations

The Cerebrospinal Fluid and Plasma Concentrations of γ -Trace and β_2 -Microglobulin at Various Ages and in Neurological Disorders

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Summary. The concentrations of γ -trace and β_2 -microglobulin in cerebrospinal fluid (CSF) and plasma were determined in 64 individuals of various ages without signs of organic disorder in the central nervous system (CNS). A strong connection was found between the CSF level of γ -trace and the age of the individual, with the CSF level of newborns being 3–4 times that of adults. A similar, but less marked, connection was found for the CSF level of β_2 -microglobulin and the age of the individual. The plasma levels of the two proteins also varied with the age of the individual, but the variations were not as great as those of the CSF levels. The results strongly emphasize the necessity of using age-matched reference values when CSF and plasma levels of the proteins are to be evaluated in different groups of patients.

Thirteen children and 98 adults with various neurological disorders were also examined. Significantly increased CSF levels of γ -trace and β_2 -microglobulin as well as increased plasma concentration of γ -trace and CSF/plasma gradient of β_2 -microglobulin were found in infectious disorders. Increased γ -trace concentration in plasma and β_2 -microglobulin concentration in CSF were seen in cerebrovascular disorders. The mechanisms which regulate the turnover of proteins in CSF are discussed.

Key words: Cerebrospinal fluid proteins (analysis) – β_2 -microglobulin – Blood-brain barrier – Cerebrospinal fluid (metabolism) – Age factors.

Zusammenfassung. Die Konzentration von γ -Trace und β_2 -Mikroglobulin wurde in Liquor und Plasma von 64 Personen unterschiedlichen Alters bestimmt, welche keine Zeichen einer organischen Störung des ZNS aufwiesen. Es zeigte sich ein enger Zusammenhang zwischen der Liquorkonzentration von γ -Trace und dem Alter der betreffenden Person, wobei Neugeborene einen 3–4fach höheren Wert als Erwachsene aufwiesen. Eine ähnliche, jedoch weniger ausgeprägte Altersabhängigkeit wurde für die Konzentration von β_2 -

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Mikroglobulin im Liquor festgestellt. Die Plasmakonzentration der beiden Proteine variierte ebenfalls je nach Alter der Person, die Schwankungen waren jedoch nicht so deutlich wie im Liquor. Diese Ergebnisse zeigen, daß es dringend erforderlich ist, für die Bestimmung der Proteine in Liquor und Plasma Kontrollwerte aus der betreffenden Altersgruppe zu verwenden.

Zusätzlich wurden 13 Kinder und 98 Erwachsene mit neurologischen Erkrankungen untersucht. Signifikant erhöhte Liquorwerte von γ -Trace und β_2 -Mikroglobulin, eine erhöhte Plasmakonzentration von γ -Trace sowie ein erhöhter Liquor/Plasma-Gradient für β_2 -Mikroglobulin wurden bei Infektionskrankheiten gefunden. Bei cerebrovasculären Erkrankungen waren die γ -Trace-Konzentration im Plasma und der β_2 -Mikroglobulin-Wert im Liquor erhöht.

Die Mechanismen der Proteinregulierung im Liquor werden diskutiert.

γ -trace or post- γ -globulin is a low molecular weight protein of 11,500 daltons [5], and was first described in 1961. The protein has been demonstrated in human cerebrospinal fluid, plasma and in the urine of patients with tubular proteinuria [4, 6, 23, 26]. The biological function of γ -trace is unknown. New methods for the analysis of γ -trace has allowed determination of its concentration in all investigated biological fluids [21]. The concentration of γ -trace in CSF in normal adults was five times that of plasma. The high CSF/plasma gradient [21] and the lack of correlation between the concentration of γ -trace and that of total proteins in CSF [20] indicated a production of γ -trace in the CNS.

The purpose of the present study was to determine the normal variation of the CSF and plasma concentrations of γ -trace in infants, children and adults, and to investigate the levels of γ -trace in some CNS disorders. The levels of β_2 -microglobulin were also analysed to allow a comparison with those of γ -trace.

Materials and Methods

Human Body Fluids

The Reference Population of Infants and Children. Paired samples of CSF and plasma were collected from 29 infants and children without apparent neurological disorder. Nine of these were newborn infants in the 37th–42nd weeks of gestation, 13 were infants 2 weeks–1 year old and 7 were 1–20 years old. All of them had a lumbar puncture as part of a bacteriological investigation to exclude septicemia or meningitis. In all of these 29 children the bacteriological cultures and CSF analyses were negative and all were considered healthy within one week.

Children with Neurological Disorders. Samples were also analysed from 13 children with disorders affecting the CNS. Six suffered from acute aseptic meningitis, 1 from meningococcal meningitis, 1 from group B streptococcal septicemia and meningitis, 1 from meningeal lymphoma and 1 from infantile myoclonic seizures. Three children later developed cerebral palsy syndromes.

The Reference Population of Adults. Samples of CSF, obtained by lumbar puncture, and plasma were collected from 35 adults (14 females and 21 males) with minor neurological complaints, but with normal total protein concentration of the CSF, normal cell count in the CSF, normal CSF/plasma gradients of albumin and IgG and normal electrophoretic pattern of the CSF. In this reference population, 16 individuals were 21–40 years old, 11 were 41–60 years old and 8 were 61–78 years old.

Adults with Neurological Disorders. Samples were also obtained from 98 adults with well defined neurological disorders.

The group of patients with multiple sclerosis (N 13) was diagnosed according to the criteria given by Schumacher et al. [32], i.e. a course with neurological symptoms indicative of multiple lesions in the central nervous system which started between 15 and 45 years of age and where the initial symptoms improved and were followed by one or more relapses. CSF protein analysis was not used to establish the diagnosis.

The group of possible multiple sclerosis (N 17) consisted of patients, who either had one attack of symptoms indicating lesions of the CNS and where the symptoms began between 15 and 45 years of age and were followed by good recovery, or patients with onset, often in the middle age, of single neurological CNS symptoms, where other neurological diseases could be excluded by appropriate investigations.

The group of patients (N 21) with cerebrovascular disorders included cerebral infarction, transitory ischemic attack (TIA), reversible ischemic neurological deficit (RIND), intracerebral hemorrhage, subdural and subarachnoid hemorrhage. The hemorrhages were all diagnosed with neuroradiological investigations including computed tomography.

The group of degenerative disorders consisted mainly of patients (N 12) with progressive dementia, where the only finding was cerebral atrophy. Patients with amyotrophic lateral sclerosis were included in the group.

The tumor group (N 6) included acoustic neuroma, intramedullary gliomas, intraspinal neuroma, meningeal carcinomatosis and Hodgkin's disease.

The infectious disorder group (N 8) consisted of patients with meningitis, encephalitis, chorioretinitis and myelitis.

Three patients suffered from systemic lupus erythematosus or arteritis with cerebral symptoms.

Three patients with the Guillain-Barré syndrome were in the acute as well as in the remission state of the disease.

The group of patients (N 15) with other neurological disorders included epilepsy, poly-neuropathy and intervertebral disc protusion.

Antisera

A specific, unabsorbed antiserum against γ -trace was produced as described [21]. An antiserum produced in rabbit against β_2 -microglobulin and peroxidase conjugated swine immunoglobulins against rabbit IgG were obtained from DAKO immunoglobulins Ltd, Copenhagen, Denmark. All other antisera were available at the laboratory.

Immunochemical Methods

The γ -trace concentration was determined by a competitive type of enzyme immunoassay [21] using pure γ -trace, isolated from the urine of a patient with tubular proteinuria, as a primary standard. The detection limit of the assay was approximately 30 $\mu\text{g/l}$. β_2 -microglobulin was quantitated by an enzyme amplified single radial immunodiffusion technique [21].

β_2 -Microglobulin Standards

A pure solution of β_2 -microglobulin with spectrophotometrically determined concentration ($\epsilon_{280} = 19,850$) was used as a primary standard and was a kind gift from Dr. Bengt G. Johansson, University of Lund.

A secondary standard consisting of uremic plasma was calibrated with the enzyme amplified single radial immunodiffusion technique against the primary standard and was used as a working standard.

Statistical Analyses

To evaluate differences between groups the Wilcoxon rank test was used. Differences were considered significant when P -values < 0.05 were obtained in the two-tailed test. Groups containing less than four individuals were excluded from the tests of probability.

Table 1. The concentrations and gradients of γ -trace and β_2 -microglobulin in age groups of the reference population

Age category	N	CSF γ -trace (mg/l) mean $\pm$ SD	Plasma γ -trace (mg/l) mean $\pm$ SD	γ -trace CSF—gradient plasma mean $\pm$ SD	CSF β_2 -microglobulin (mg/l) mean $\pm$ SD	Plasma β_2 -microglobulin (mg/l) mean $\pm$ SD	β_2 -microglobulin CSF—gradient plasma mean $\pm$ SD	γ -trace gradient β_2 -m gradient mean $\pm$ SD
Newborn								
37–42 weeks of gestation	9	18.5 $\pm$ 2.3	3.4 $\pm$ 1.4	6.2 $\pm$ 1.7	1.9 $\pm$ 0.52	3.9 $\pm$ 1.7	0.53 $\pm$ 0.15	12.6 $\pm$ 2.1
2– 4 weeks	4	19.1 $\pm$ 3.8	2.6 $\pm$ 0.47	7.1 $\pm$ 0.50	2.2 $\pm$ 0.44	4.4 $\pm$ 2.4	0.60 $\pm$ 0.22	15.8 $\pm$ 5.2
1– 3 months	2	11.6 $\pm$ 0.95	2.6 $\pm$ 0.37	4.6 $\pm$ 1.0	1.9 $\pm$ 0.17	6.6 $\pm$ 2.3	0.33 $\pm$ 0.09	14.2 $\pm$ 0.61
3–12 months	7	4.2 $\pm$ 1.5	1.6 $\pm$ 0.55	2.9 $\pm$ 1.6	0.65 $\pm$ 0.19	2.3 $\pm$ 0.69	0.30 $\pm$ 0.12	9.5 $\pm$ 2.9
1–20 years	7	3.6 $\pm$ 0.97	1.1 $\pm$ 0.21	3.2 $\pm$ 1.1	0.60 $\pm$ 0.18	1.7 $\pm$ 0.64	0.37 $\pm$ 0.12	8.8 $\pm$ 2.2
21–40 years	16	5.0 $\pm$ 0.99	0.90 $\pm$ 0.15	5.6 $\pm$ 1.2	0.88 $\pm$ 0.27	1.2 $\pm$ 0.23	0.74 $\pm$ 0.23	8.1 $\pm$ 2.5
41–60 years	11	5.2 $\pm$ 1.9	1.0 $\pm$ 0.17	5.1 $\pm$ 1.7	1.0 $\pm$ 0.18	1.4 $\pm$ 0.23	0.73 $\pm$ 0.12	7.0 $\pm$ 1.9
> 60 years	8	6.6 $\pm$ 1.6	1.5 $\pm$ 0.42	4.7 $\pm$ 1.8	1.3 $\pm$ 0.30	2.0 $\pm$ 0.39	0.66 $\pm$ 0.23	7.2 $\pm$ 1.9
All adults > 21 years	35	5.4 $\pm$ 1.6	1.1 $\pm$ 0.34	5.2 $\pm$ 1.6	1.0 $\pm$ 0.30	1.5 $\pm$ 0.42	0.72 $\pm$ 0.21	7.5 $\pm$ 2.3

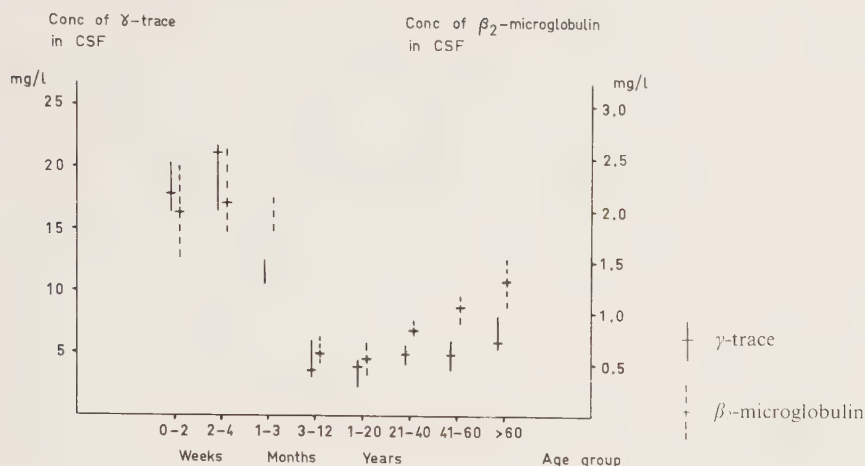


Fig. 1. The concentrations in CSF of γ -trace and β_2 -microglobulin at various ages are demonstrated in individuals without organic neurological disorder. The median and the values between the lower and higher quartiles (25th and 75th percentiles) are shown for each age group, except for the age group of 1-3 months, where the complete range is shown

Results

Correlations of γ -Trace and β_2 -Microglobulin Levels

In a reference population consisting of both children and adults the CSF and plasma concentrations of γ -trace and β_2 -microglobulin were determined. The correlation between the concentrations of the two proteins was in CSF $r=0.84$ and in plasma $r=0.76$.

Variation with Age

In infants less than one month old the CSF concentration of γ -trace was three times, and that of β_2 -microglobulin twice the adult level (Table 1 and Fig. 1). The CSF/plasma gradients of both γ -trace and β_2 -microglobulin were high in young infants, but fell to low values in older infants. The proportion between the CSF/plasma gradient of γ -trace and that of β_2 -microglobulin (gradient of γ -trace/gradient of β_2 -microglobulin) was greatest in infants less than 3 months old ($P<0.01$) compared with all adults in the reference populations. In individuals older than 60 years the concentrations of γ -trace and β_2 -microglobulin in CSF were slightly increased.

Values in Infants with Neurological Disorders

Eight children with meningitis and five with other neurological disorders were examined (Table 2). A remarkably high γ -trace concentration in CSF (40.8 mg/l)

Table 2. The concentrations and gradients of γ -trace and β_2 -microglobulin in individual infants with neurological disorders

Diagnosis	Age	CSF γ -trace (mg/l)	Plasma γ -trace (mg/l)	γ -trace CSF plasma gradient	CSF β_2 -microglobulin (mg/l)	Plasma β_2 -microglobulin (mg/l)	β_2 -microglobulin CSF plasma gradient
Cerebral palsy	Newborn	20.4	2.7	7.6	2.4	6.1	0.39
Cerebral palsy	Newborn	19.6	4.7	4.2	1.8	—	—
Septicemia and meningitis	Newborn	40.8	3.8	10.9	2.1	5.0	0.42
Aseptic meningitis	1 week	8.7	3.6	2.4	2.4	—	—
Cerebral palsy	1.6 months	18.4	2.5	7.6	2.8	4.5	0.62
Aseptic meningitis	1.7 months	16.4	2.1	7.9	5.0	3.5	1.4
Aseptic meningitis	4 months	11.9	2.7	4.4	1.2	4.5	0.27
Aseptic meningitis	4.6 months	2.3	1.6	1.5	0.76	2.4	0.32
Meningococcal meningitis	4.9 months	4.2	2.3	1.8	3.2	1.9	1.7
Infantile myoclonic seizures	7.6 months	5.8	1.4	4.2	0.50	2.6	0.20
Meningeal lymphoma	11.3 months	7.5	0.95	7.9	0.93	0.83	1.1
Aseptic meningitis	1.1 year	4.0	1.6	2.5	0.74	2.3	0.33
Aseptic meningitis	13.7 years	6.8	1.6	4.3	2.3	1.7	1.3

Table 3. The concentrations and gradients of γ -trace and β_2 -microglobulin in groups of adults suffering from various neurological disorders

Patient category	N	CSF γ -trace (mg/l) mean $\pm$ SD	Plasma γ -trace (mg/l) mean $\pm$ SD	γ -trace CSF plasma gradient mean $\pm$ SD	CSF β_2 -microglobulin (mg/l) mean $\pm$ SD	Plasma β_2 -microglobulin (mg/l) mean $\pm$ SD	β_2 -microglobulin CSF plasma gradient mean $\pm$ SD
Multiple sclerosis	13	5.0 $\pm$ 2.2	1.0 $\pm$ 0.15	4.9 $\pm$ 2.0	1.1 $\pm$ 0.35	1.3 $\pm$ 0.25	0.86 $\pm$ 0.23
Possible multiple sclerosis	17	5.3 $\pm$ 1.4 N=21	1.1 $\pm$ 0.36 N=15	5.4 $\pm$ 2.2 N=15	1.3 $\pm$ 0.90 N=21	1.6 $\pm$ 0.56 N=15	0.82 $\pm$ 0.37 N=15
Cerebrovascular disorders		7.4 $\pm$ 3.3	1.9 $\pm$ 0.71***	4.4 $\pm$ 1.9	1.5 $\pm$ 0.53*	2.3 $\pm$ 1.2	0.78 $\pm$ 0.45
Degenerative disorders	12	7.3 $\pm$ 5.5 N=6	1.4 $\pm$ 0.20 N=3	5.0 $\pm$ 3.0 N=3	1.5 $\pm$ 1.1 N=6	1.9 $\pm$ 0.34 N=3	0.78 $\pm$ 0.50 N=3
Tumors		7.3 $\pm$ 4.3	1.6 $\pm$ 0.51	7.9 $\pm$ 3.8	2.3 $\pm$ 2.1	2.1 $\pm$ 0.87	2.3 $\pm$ 2.3
Infectious disorders	8	6.9 $\pm$ 1.8**	1.4 $\pm$ 0.29**	5.1 $\pm$ 1.4	1.5 $\pm$ 0.51***	1.7 $\pm$ 0.27	0.94 $\pm$ 0.30**
Immunological disorders	3	4.6 $\pm$ 1.8	1.6 $\pm$ 0.76	3.3 $\pm$ 1.9	1.6 $\pm$ 0.74	2.8 $\pm$ 1.9	0.68 $\pm$ 0.29
Guillain-Barré syndrome	3	5.0 $\pm$ 1.2	1.3 $\pm$ 0.15	3.9 $\pm$ 1.2	0.81 $\pm$ 0.06	1.6 $\pm$ 0.58	0.58 $\pm$ 0.25
Other neurological disorders	15	5.0 $\pm$ 1.1	1.0 $\pm$ 0.16	5.2 $\pm$ 1.3	1.0 $\pm$ 0.30	1.5 $\pm$ 0.32	0.75 $\pm$ 0.18

Levels of significance: * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$

was found in a newborn infant with group B streptococcal septicemia and meningitis. The CSF/plasma gradient of β_2 -microglobulin was above 1.0 in three children with meningitis and in one with meningeal lymphoma.

Values in Adults with Neurological Disorders

The values of the disorder groups (Table 3) were compared with those of age matched reference populations.

γ -Trace Values. A significantly ($P < 0.02$) increased CSF concentration of γ -trace was found in the group of infectious disorders. The plasma concentration of γ -trace was increased in the groups of cerebrovascular ($P < 0.01$) and infectious ($P < 0.02$) disorders. However the CSF/plasma gradient of γ -trace did not differ significantly from the values of the reference populations.

β_2 -Microglobulin Values. Increased CSF concentration of β_2 -microglobulin was found in the groups of cerebrovascular ($P < 0.05$) and infectious ($P < 0.01$) disorders. The CSF/plasma gradient of β_2 -microglobulin was increased ($P < 0.02$) in the group of infectious disorders.

Time Dependent Variations

A patient with cerebral infarction underwent lumbar puncture every second day for 3 weeks. The γ -trace concentration of all the CSF samples were within 1 SD of the mean of the reference group and showed no increasing or decreasing trend.

Discussion

Regulating Mechanisms of the CSF Composition

The concentrations in CSF of γ -trace and β_2 -microglobulin (Fig. 1) as well as other proteins [35, 37] are related to the age of the individual. The age influenced mechanisms, which regulate the turnover of proteins in the CSF, are:

The formation, flow and bulk absorption of CSF [14, 18, 25, 28, 31].

The permeability of the blood-CSF barrier [13, 17], the hydrodynamic radii [9] and the charge [3] of the proteins.

The plasma concentration of proteins and their molecular organisation (e.g. as monomers or complexes).

The release of proteins locally synthesized within the CNS [10, 11, 12, 19, 31, 34].

The CSF/plasma gradients or ratios of albumin, α_2 -macroglobulin and IgG are used as clinical parameters of the condition of the different protein regulating mechanisms [10, 11, 19, 30, 31, 35].

A passive size dependent filtration from plasma to CSF of γ -trace and β_2 -microglobulin, which have similar hydrodynamic radii [36], would have resulted in CSF/plasma gradients less than 0.05 [9]. The mean gradients of the adult reference population (γ -trace 5.2 and β_2 -microglobulin 0.72) were far above this value, which makes a local production in the CNS of the two proteins likely.

Regional Differences of the Composition of the CSF Within the Subarachnoid Space

The concentrations of most proteins in the CSF are higher in lumbar fluid than in ventricular fluid. Different proteins demonstrate different changes in the concentration. Lumbar fluid contains 1.6 times more total protein and 2.2 times more albumin than ventricular fluid in adult individuals [39]. All samples of CSF described in this paper were taken by lumbar puncture. The concentrations of γ -trace and β_2 -microglobulin in ventricular and cisternal fluid remain to be determined.

Correlations

There was a correlation between the γ -trace and the β_2 -microglobulin concentrations in both CSF ($r=0.84$) and in plasma ($r=0.76$) in the combined reference group of children and adults. This indicates that some mechanisms regulating their turnover are in common.

Influence of Age

The low rate of production, flow and bulk absorption of CSF in very young [28] and old [15] individuals results in a synchronous increase of the concentration of proteins in the CSF [31].

The maturing blood-CSF barrier, the increasing flow and absorption of CSF and the increasing glomerular filtration rate may contribute to explain the changes in the concentrations and the gradients of the two proteins in growing individuals.

The high concentration of γ -trace in CSF in the first month of life, when the blood-CSF barrier is not fully mature [37], and the high CSF/plasma gradient of γ -trace in relation to the gradient of β_2 -microglobulin indicated a higher production of γ -trace within the CNS of young infants than within the CNS of other age groups.

The plasma values of β_2 -microglobulin in newborn infants presented in this investigation are in agreement with earlier findings [1], which demonstrate high concentrations due to a high production in newborn prematures in relation to their glomerular filtration rate. Proteins of low molecular weight such as γ -trace and β_2 -microglobulin are catabolized by the kidneys [2, 21, 24], and the plasma concentration of β_2 -microglobulin, the glomerular filtration rate and the plasma concentration of creatinine are correlated [16, 38].

Children with Neurological Disorders

In the meningitis group both high and low γ -trace values were found, when compared with age matched reference values.

Three children with meningitis and one with meningeal lymphoma had a CSF/plasma gradient of β_2 -microglobulin above 1.0 suggesting a high intrathecal production of β_2 -microglobulin or an impaired bulk absorption of CSF.

γ -Trace Values in Adults with Neurological Disorders

Increased concentration of γ -trace in CSF but normal CSF/plasma gradient were found in the group of infectious disorders.

In contrast to earlier reports [22, 27] no significant decrease in the concentration of γ -trace in CSF was found in the group of multiple sclerosis. In the earlier investigations the age of the reference population and of the disorder group were not reported. Different mean age of the groups may influence the results obtained, as shown in this work.

Only small fluctuations in the concentration of γ -trace were observed when a patient in the cerebrovascular group of disorders was examined repeatedly over a period of 3 weeks. This indicates a low day to day variation in the level of γ -trace in CSF for this group of disorder.

The ranges of the concentrations of γ -trace in CSF in the groups of cerebrovascular and degenerative disorders and in the group of tumors were wide. A small number of patients had concentrations of γ -trace in CSF above 10 mg/l. A man with calcifications around the foramina of Monro demonstrated a concentration of γ -trace of 22 mg/l. Two patients with space occupying tumors affecting the CSF flow and absorption had high concentrations of γ -trace in the CSF. This suggests that patients with impaired CSF flow and absorption may have abnormally high levels of γ -trace in the CSF.

β_2 -Microglobulin Values in Adults with Neurological Disorders

The CSF/plasma gradient of β_2 -microglobulin was significantly increased in the group of infectious disorders compared with the age-matched reference values. This observation is in agreement with previous findings [12, 34]. It has been reported that the production and the concentration of β_2 -microglobulin in plasma are increased in some inflammatory and malignant disorders outside the CNS [7, 8, 33]. If the activity of such or similar disorders is higher within than outside the CNS, the intrathecal production of β_2 -microglobulin and the CSF/plasma gradient will increase [12, 29, 34]. An impaired flow and bulk absorption of CSF may also contribute to an increased concentration of β_2 -microglobulin in CSF in infectious and cerebrovascular disorders.

Conclusion

The determination of the γ -trace and β_2 -microglobulin concentrations and the CSF/plasma gradients may contribute to the understanding of the mechanisms which regulate the composition of the CSF and which are disturbed in various disorders. Since the normal dynamic steady state of CSF varies with age, it is important to use age-matched reference values, when the concentration of these proteins in the CSF are to be investigated in various disease states.

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HUMAN BRAIN CORTICAL NEURONS CONTAIN γ -TRACE. RAPID ISOLATION, IMMUNOHISTOCHEMICAL AND PHYSICOCHEMICAL CHARACTERIZATION OF HUMAN γ -TRACE

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ABSTRACT

Human γ -trace was isolated from urine by a rapid two-step procedure comprising pressure ultrafiltration and ion-exchange chromatography. Immunohistochemical studies of human cerebral tissue demonstrated that γ -trace occurs in the cortical neurons. The sequence of the 37 amino-terminal amino acid residues was determined. Sequence similarities were found between γ -trace and human glucagon and between γ -trace and porcine CCK indicating that γ -trace might be related to the peptidergic GEP neuroendocrine system.

KEY WORDS γ -trace / neuropeptides / cortical neurons

γ -Trace is an alkaline, low molecular weight protein, first described in 1961 as a protein in cerebrospinal fluid (CSF) and in urine from patients with renal failure (2, 4, 23). The biological function of γ -trace is unknown although the protein has been demonstrated to occur not only in human CSF and urine but also in plasma, saliva and semen (3, 5, 10). Recently, a sensitive enzyme immunoassay for the measurement of γ -trace was described which allowed quantitation of the protein in all human fluids investigated. It was found that in healthy adults the concentration of γ -trace in CSF is 5.5 times that in plasma (18). It was also observed that the CSF level of newborns was 3-4 times the CSF level of adults

(19). These observations prompted studies concerning the presence of γ -trace in some cell types of the central nervous system (CNS) and also structural studies concerning possible similarities with other proteins or peptides known to occur in the CNS. This work describes a rapid isolation of human γ -trace and demonstrates its presence in human neurons by an immunohistochemical method. The amino-terminal amino acid sequence shows some similarities with those of the gastroenteropancreatic (GEP) neurohormones glucagon and cholecystokinin (CCK).

MATERIALS AND METHODS

Reagents Diethylpyrocarbonate (Pyrokohlen-säurediäthylester) and *p*-benzoquinone were obtained from Merck, Darmstadt, Germany and sodium borohydride and 3,3'-diaminobenzidine tetrahydrochloride from British Drug Houses Ltd., Poole, England. Reagents and solvents for the sequencer (sequencer grade) were from Rathburn Chemicals Ltd., Walker-

Abbreviations: CSF, cerebrospinal fluid; CNS, central nervous system; GEP, gastroenteropancreatic; CCK, cholecystokinin; PAP, soluble complexes of horseradish peroxidase / rabbit anti-horseradish peroxidase; PBS, 0.05 M phosphate buffer, pH 7.4 with 0.1 M NaCl; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate

burn, Peeblesshire, Scotland and 10% SP-400 on 100/120 mesh Chromosorbe-WHP from Supelco Inc., Bellefonte, Pa. DEAE-Sephadex A-50, activated CH-Sepharose 4B, Sephadex G-50 Superfine and Sephadex G-10 were from Pharmacia Fine Chemicals, Uppsala, Sweden and Ultrogel AcA 202 from LKB-produkter AB, Bromma, Sweden. Glucagon was purchased from Novo Industri A/S, Bagsvaerd, Denmark and Cholecystokinin₃₉ was a kind gift from Professor V. Mutt, Karolinska Institutet, Stockholm, Sweden. Complete Freund's Adjuvant was obtained from Difco Laboratories, Detroit, Mich. All other chemicals were of reagent grade.

Antisera Swine antiserum against rabbit IgG, soluble complexes of horseradish peroxidase/rabbit anti-horseradish peroxidase (PAP) and peroxidase conjugated swine immunoglobulins against rabbit IgG were all obtained from DAKO immunoglobulins Ltd., Copenhagen, Denmark.

Isolation of γ -Trace

Urine collection Urine from uremic patients was collected in bottles containing NaN_3 as preservative. After 1–3 days at room temperature the urine was either frozen or used directly for isolation of γ -trace.

Urine concentration The urine was filtered by passing through a Hollow Fiber Dialyser Concentrator DC2 (Amicon Co., Lexington, Ms.) equipped with a H1P 10 capillary filter (nominal retention limit: 10,000 daltons). The ultrafiltrate was collected and concentrated by repeated ultrafiltration but this time with a H1P 5 capillary filter (nominal retention limit: 5,000 daltons). The concentrate was desalted by gel chromatography on a column of AcA 202.

Ion-exchange chromatography A column of DEAE-Sephadex A-50 (5×52 cm) was used to isolate γ -trace from 12–15 litres of urine concentrated to about 50 ml. The column was eluted at 6°C with 0.05 M Tris-HCl buffer, pH 8.55, and at a flow rate of 25 ml per hr. Fractions of 7 ml were collected and examined with agarose gel electrophoresis (14), double radial immunodiffusion (26) using a monospecific antiserum against γ -trace and with light absorption at 280 nm. The fractions were pooled and desalted by passage through a column of Sephadex G-10.

Production of Anti- γ -Trace Antiserum

The purified material obtained from the ion-exchange chromatography (Pool 1) was chromatographed on a column of Sephadex G-50 Superfine and the mid-fraction from the resulting single symmetric protein peak was used for immunization of 3 rabbits. Three mg of γ -trace in 1.5 ml 0.15 M NaCl was emulsified with 1.5 ml complete Freund's Adjuvant and injected into the hind paws as well as subcutaneously in different parts of the back. Three weeks later identical booster injections were given and thereafter the rabbits were bled every 2 weeks.

Immunohistochemical Technique

Tissue specimens Biopsy specimens were obtained from apparently normal cerebral cortex in the vicinity of a tumor in 3 patients undergoing neurosurgery for astrocytoma or meningioma. Within 5 min of removal the specimens, which were 3–4 mm across, were quickly frozen in a propane-butane mixture to the temperature of liquid nitrogen and lyophilized for 8 days. Tissues from the CNS were also taken from other patients at autopsy 6–12 hr postmortem.

Fixation The freeze-dried tissue was vapour-fixed in diethylpyrocarbonate at 60°C for 3 hr (27) and vacuum embedded in paraffin. Sections were cut at 6 μm , mounted on glasses and air dried for 1 hr in front of a fan. After deparaffinization, some sections were post-fixed in 3% (w/v) *p*-benzoquinone in 0.05 M phosphate buffer, pH 7.4 with 0.1 M NaCl (PBS) for 3 min, washed in PBS for 3×1 min and treated with 1% (w/v) NaBH_4 in PBS for 10 min to reduce remaining active fixative.

Staining The Sternberger peroxidase-antiperoxidase (PAP) technique was used with hydrogen peroxide and diaminobenzidine tetrahydrochloride as enzyme substrate. Hypertonic buffers containing 0.5 M NaCl and Triton-X 100 were used for dilutions and most washing steps (31), but no such step was allowed to exceed 1 min. The working dilution of the first antiserum against γ -trace was 1:300.

Controls Absorbed antiserum and serum from a non-immunized rabbit were used as negative controls. All working dilutions were 1:300

Antiserum absorption The specific antibodies against γ -trace were removed from the antiserum by pure γ -trace coupled to a CH-Sepharose column equilibrated with 0.05 M Tris-HCl buffer, pH 7.4 containing 0.1 M NaCl. The

absorbed antiserum was concentrated to the same protein concentration as the unabsorbed antiserum. The specific antibodies against γ -trace were recovered by elution of the column with 0.1 M sodium acetate, pH 3.5, containing 0.5 M NaCl and with 0.1 M glycine, pH 2.2, containing 0.5 M NaCl. The immunological activity against γ -trace in each fraction was tested by double radial immunodiffusion and by immunohistochemical tests with the PAP technique. The antiserum was also absorbed by incubation overnight with glucagon or CCK₃₉ in molar amounts corresponding to four times the precipitating titre of the antiserum.

Amino Acid Analyses

Lyophilized salt-free samples of reduced and carboxy-methylated γ -trace were hydrolyzed for 24, 48 and 72 hr at 110°C in 6 M HCl in evacuated, sealed Pyrex tubes. The amino acids of the hydrolyzates were analyzed with a one-column system on a Kontron Liquimat III amino acid analyzer with the Durrum DC-6A resin.

Amino Acid Sequence Analyses

Automated amino acid sequence analysis by the method of Edman and Begg (8) was carried out on a Beckman 890 C Sequencer using the program of Thomsen *et al.* (33) in which the buffer is added before the phenylisothiocyanate to reduce background accumulation. All phenylthiohydantoin (PTH) amino acids were identified by high performance liquid chromatography on a 30-cm Waters u-Bondapak C18 column using stepwise elution with methanol-containing buffers for the ethyl acetate phases (Waters Associates Inc., Milford, Ma., Internal communication 03-LS-11-76) and acetonitrile-containing buffers for the aqueous phases (36). All PTH-amino acids were also identified by thin layer chromatography (13) and some were also determined by gas-liquid chromatography according to Pisano *et al.* (28) using a Varian 2400 instrument with a column of 10% SP-400 on Chromosorb-WHP.

Miscellaneous Methods

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (15) and heated Biuret-Folin protein assay (6) were performed as described. The concentration of γ -trace was

determined by an enzyme amplified single radial immunodiffusion technique (19). The samples were diluted by a solution of 10% (v/v) non-immunized rabbit serum in 0.025 M barbital buffer, pH 8.6. The freeze-drier for processing tissue was purchased from Lynhagen Electronics, Furulund, Sweden.

RESULTS

Isolation

On agarose gel electrophoresis of fresh concentrated human CSF or urine γ -trace appears as a narrow protein band closely cathodally to the immunoglobulins (Fig. 1). Agarose gel electrophoresis was therefore used to monitor the earlier attempts to isolate γ -trace. Later, when a monospecific antiserum against γ -trace became available, all isolation procedures were demonstrated both by agarose gel electrophoresis and by use of the antiserum. A simple two-step procedure was found to be sufficient for isolating γ -trace from urine from patients with tubular proteinuria.

In the first step the original urine was passed through a capillary filter allowing passage

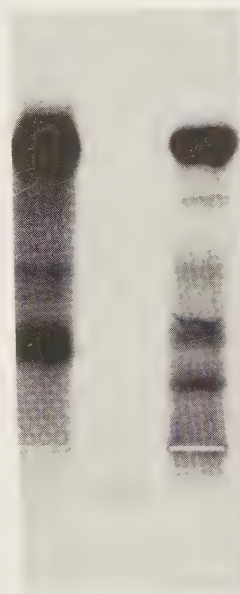


Fig. 1 Agarose gel electrophoresis at pH 8.6 of (from left to right) fresh concentrated urine from uremic patients, isolated γ -trace and normal human plasma

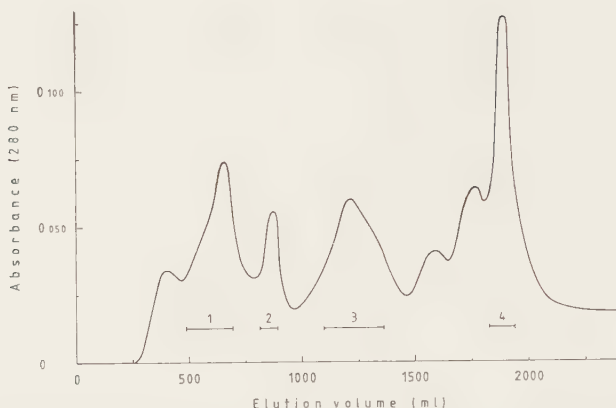


Fig. 2 Chromatography on DEAE-Sephadex of urinary proteins after concentration by double ultrafiltration. The light absorption at 280 nm of each fraction was measured with a Cary 219 spectrophotometer (Varian Ass., Palo Alto, Cal.). Fractions reacting with a monospecific anti- γ -trace antiserum in double radial immunodiffusion were collected in 4 pools. Pool 1 was pure and contained 8.7 mg γ -trace as measured by enzyme amplified single radial immunodiffusion. Pools 2-4 contained 0.6, 5.9, and 0.2 mg γ -trace, respectively, but all three were contaminated with other urinary proteins.

of a large part of γ -trace but retaining most of the high molecular weight urinary proteins (Table 1). The ultrafiltrate was then highly concentrated by passage through a capillary filter with a low molecular weight cut-off, dialyzed against 0.05 M Tris-HCl buffer, pH 8.55, and fractionated on a column of DEAE-Sephadex A-50 in the same buffer. The elution diagram and the pooling of fractions are shown in Fig. 2. Although four of the light-absorbing peaks contained material reacting with an antiserum against γ -trace on double radial immunodiffusion, only one of the pools (1 in Fig. 2) contained pure γ -trace as evidenced by SDS polyacrylamide gel electrophoresis. In addition, pool 1 was the only one which on agarose gel electrophoresis gave rise to a protein band in the original cathodal position of γ -trace (Fig. 1). The yields of the different steps in the isolation of γ -trace are given in Table 1.

Physico-Chemical Properties

SDS polyacrylamide gel electrophoresis of the unreduced as well as of the reduced protein only produced a single narrow band. With the use of H- and L-chains of IgG, human prealbumin and β_2 -microglobulin as molecular

weight markers, the molecular weight of the reduced protein was estimated to be 12,600 daltons.

The amino acid composition of γ -trace is given in Table 2. No galactosamine or glucosamine could be detected.

Several runs of native, reduced and ^{14}C -carboxymethylated γ -trace in the sequenator revealed the amino-terminal amino acid sequence shown in Fig. 3. The figure also demonstrates the amino acid sequences of human glucagon and of the carboxyl-terminal half of CCK. Glucagon shows a sequence similarity with γ -trace comprising 7 of its 29 amino acid residues. Three of the five carboxyl-terminal residues of CCK are found in the sequence of γ -trace.

Specificity of the Antiserum

When the antiserum against γ -trace was tested by classical and crossed immunoelectrophoresis with the use of pure γ -trace and several dilutions of CSF and of normal and uremic human plasma, only one precipitation arc appeared. When the antiserum was used in a competitive type of enzyme immunoassay for quantitation of γ -trace, the dose response curves for pure γ -trace, normal human CSF and normal and

Table 1 *Isolation of Human γ -Trace*

	Total protein ^a (mg)	γ -Trace ^b (mg)	Yield (%)	Purification (-fold)
Original urine pool	23,850	65.7	100	1
Double pressure ultrafiltration	2,100	22.9	34.9	4.0
DEAE-Sephadex chromatography	8.7	8.7	13.2	363

^a Measured by heated Biuret-Folin assay.^b Measured by enzyme amplified single radial immunodiffusion.Table 2 *Amino Acid Composition of Human γ -Trace*

Constituent	Residues per molecule ^a	To nearest integer
Aspartic acid	11.1	11
Threonine ^b	6.8	7
Serine ^b	7.5	8
Glutamic acid	11	11
Proline	7.6	8
Glycine	8.6	9
Alanine	9.2	9
Half-cystine ^c	3.4	3
Valine ^d	9.3	9
Methionine	2.2	2
Isoleucine	1.9	2
Leucine	7.6	8
Tyrosine	3.5	4
Phenylalanine	4.7	5
Lysine	6.4	6
Histidine	2.7	3
Arginine	7.4	7
Tryptophan ^e	0.7	1
Total		113

Unless otherwise stated, all figures are average values obtained from duplicate analyses of samples hydrolyzed for 24, 48 and 72 hr.

^a Calculated on the basis of 11 residues of glutamic acid per molecule.

^b Values obtained by extrapolation to zero hr hydrolysis.

^c Determined as carboxymethylcysteine.

^d Seventy-two hr hydrolysis value only.

^e Determined spectrophotometrically (7).

uremic human plasma were parallel (19). The precipitating titre according to Becker (1) was 0.19 mg/ml.

When the antiserum absorbed with insolubilized γ -trace was tested by double radial immunodiffusion against γ -trace, no immunoprecipitates were obtained. The specific antibodies, absorbed to the affinity column, were recovered by elution at low pH and formed distinct precipitation arcs.

The immunoreactive end-point of serial dilutions of the antiserum raised against γ -trace and used in the first step of the PAP

technique was 1:2,400. The corresponding values for the solutions of specific antibodies recovered from the affinity column were 1:2,400 (antibodies eluted at pH 3.5, 2.6 g IgG/l) and 1:300 (antibodies eluted at pH 2.2, 1.9 g IgG/l).

Almost identical immunohistoactivity of neurons in cerebral tissue was obtained when the unabsorbed anti- γ -trace antiserum and the same antiserum absorbed with glucagon or CCK were used in the PAP technique. When the antiserum absorbed with γ -trace and serum from a non-immunized rabbit were used in the PAP technique, no

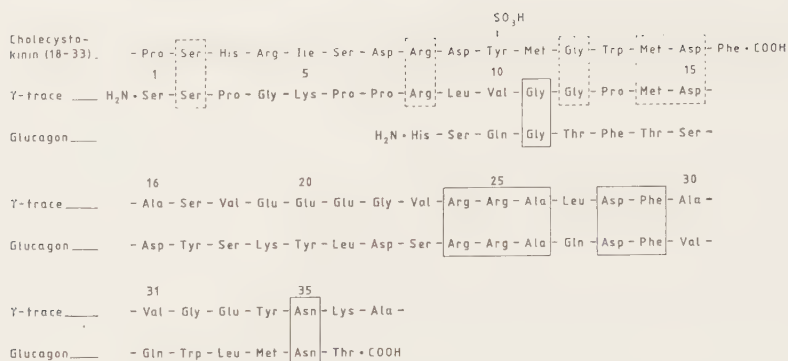


Fig. 3 The amino acid sequence of the amino-terminal end of human γ -trace. The complete amino acid sequence of human glucagon (34) and that of porcine CCK₁₈₋₃₃ (25), are also given for comparison. Identical residues of the molecules are shown in boxes.

immunoreactivity was observed.

Immunohistochemical Staining

Neurons in the cortex of the frontal and temporal lobes were immunostained, when the antiserum raised against γ -trace was used in the first step of the PAP technique (Fig. 4). The cytoplasm, as well as the dendrites, particularly the apical ones, and the nuclei, were immunoreactive. Sections, counterstained with cresyl violet, showed that most neurons were immunostained in areas where the immunohistochemical results were technically adequate. Some astrocytes were faintly stained, but many of them, as well as of the neuronal satellite glial cells were negative. The cortical neuropil in the background stained diffusely and weakly. The vessel walls, erythrocytes and leucocytes showed non-immunological peroxidase staining. In the superficial white matter neither astrocytes nor oligodendroglial cells were immunoreactive. Several sections stained better when post-fixed in *p*-benzoquinone. Necropsy specimens of brains were not immunoreactive when the antiserum against γ -trace was used.

DISCUSSION

The procedure for isolation of γ -trace described here differs from those on record (3, 11, 17, 21, 35) in that it requires only two steps; viz. ultrafiltration using two different filters and ion-exchange chromatography using DEAE-Sephadex. The yield was lower than that

reported for the isolation procedure of Čejka and Fleischmann (3) but the discriminative power of our method allows distinction between various forms of γ -trace. The most basic form of γ -trace was obtained in a pure state, while the three more acidic forms were contaminated with other urinary proteins. The most basic form of γ -trace is probably the parent molecule of the three more acidic forms since this is the form reported to be present in fresh urine and CSF and since degradation of this molecule to more acidic derivatives occurs on ageing of urine and CSF (12, 22, 35). In addition the basic form of γ -trace was the preponderant form in the yield obtained with our method.

The physico-chemical properties (molecular weight, amino acid composition, electrophoretic mobility) of the basic γ -trace isolated in this study were similar to those on record (3, 35). Only one report (Tonelle *et al.* (35)) of some physico-chemical properties of the more acidic derivatives of γ -trace is available. The report described the amino-terminal amino acid residues of three molecular species of γ -trace. One of the species was thought to be the original basic γ -trace molecule; the other two more acidic species proteolytic derivatives of the original molecule. The three amino-terminal amino acid residues reported occur as residues numbers 5, 8, and 9 in the amino acid sequence of the γ -trace molecule isolated by us. Moreover, assuming that the three molecular species differ only in the extension of their amino-terminal parts, they will differ in charge according to their electrophoretic mobilities. The most basic and

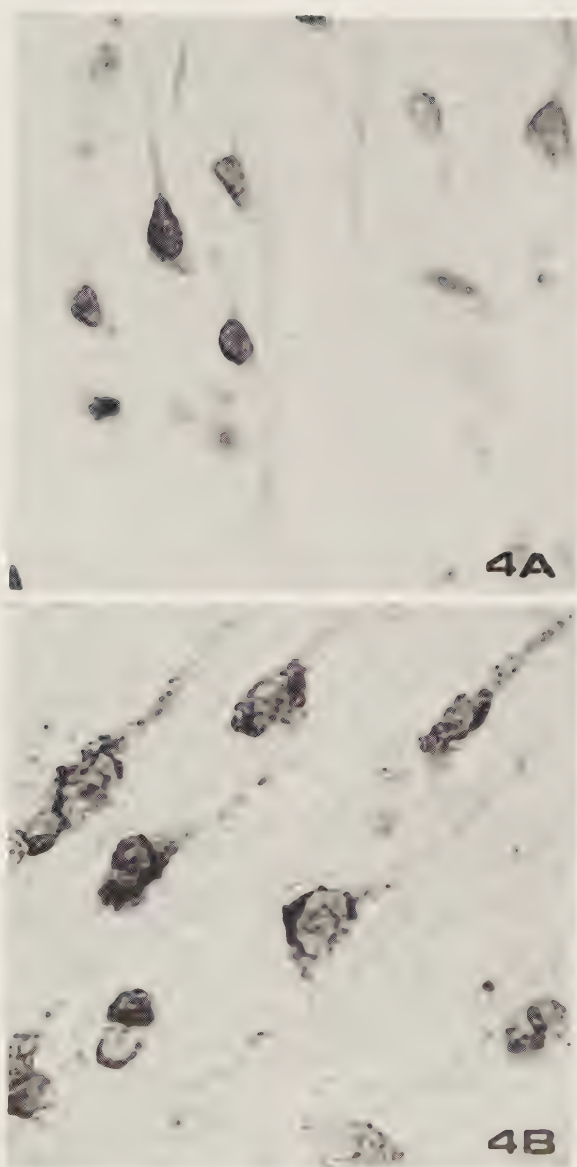


Fig. 4 Cerebral cortex with immunostaining of γ -trace in neurons, including their nuclei, cytoplasm and dendrites. Especially the apical dendrites (a) but also the basal ones (b) stained. Only an occasional glial cell was immunoreactive. There was a weak, diffuse staining of the neuropil. PAP technique a, $\times 500$; b, $\times 750$

original γ -trace molecule in the work of Tonelle *et al.* (35) is apparently devoid of the four amino terminal residues of the γ -trace species isolated by us. Whether the basic γ -trace molecule isolated in the present work is the original unaltered one emanating from cells, or whether it is a proteolytic product of a still larger molecule remains to be investigated.

The amino acid sequences of γ -trace, human glucagon and porcine CCK suggest some relationship between γ -trace and the peptidergic GEP neuroendocrine system. It should be observed that the similarity between γ -trace and CCK, although less than that between γ -trace and glucagon, is found mainly in the hormonally active C-terminal octapeptide of CCK. Further similarities between γ -trace and polypeptides of the GEP neuroendocrine system might be revealed when more is known about the primary structure of γ -trace. Preliminary results of investigations in progress show additional similarities.

That γ -trace really is associated with human neuronal cells was corroborated by the immunohistochemical studies demonstrating γ -trace in the neurons of fresh biopsy specimens of cerebral cortex from 3 patients. In the elaboration of the technique some difficulties were encountered. It was found, for example, that brain tissue obtained at autopsy was invariably negative, probably because of autolysis or diffusion of the protein out of the neurons. Only when fresh, quickly frozen small tissue specimens were obtained without diathermy could the neuronal content of γ -trace be demonstrated.

The presence of γ -trace in neurons of the human CNS is consistent with the observed high value, 5.5, of the CSF/plasma gradient for γ -trace in healthy adults (18). The weak but positive background immunoreactivity of the neuropil may be explained by the presence of γ -trace in the neuronal processes and in some glial cells as well as its presence in the extracellular cerebral fluid, draining into the CSF cavities. The extracellular cerebral fluid and the cerebrospinal fluid are known to be very similar in chemical composition (24).

Recent immunohistochemical studies using the antiserum against human γ -trace described in this work demonstrate the presence of γ -trace also in monkey and rat brain.

It is noteworthy that glucagon-like immunoreactivity and CCK also have been demonstrated in neuronal cells of the CNS. High concentrations of CCK have been reported in the

cerebral cortex (16, 29, 30), while glucagon related peptides occur mainly in nerve fibers of the thalamus and hypothalamus (20, 32). Phylogenetically, the GEP hormones seem to appear earlier in the CNS than in the alimentary tract (9). A comparison of the distribution in the CNS of γ -trace and the GEP neuroendocrine peptides might provide a clue to their possible interrelation.

The amino acid sequenator was skillfully operated by Mr Roland Nilsson. The authors also gratefully acknowledge the excellent technical assistance of Ms Veronica Grimsberg, Elise Nilsson and Christina Möller. The biopsy specimens were obtained in collaboration with Dr Lars-Göran Strömblad, Department of Neurosurgery, University Hospital, Lund. This work was supported by grants from Svenska Sällskapet för Medicinsk Forskning, Svenska Läkaresällskapets Forskningsfond, Konung Gustaf V:s 80-årsfond, Neurologiskt Handikappades Riksförbund, Anna Lisa och Sven-Eric Lundgrens stiftelse för medicinsk forskning, the Medical Faculty, University of Lund, and the Swedish Medical Research Council (Project No. B81-13X-05196-04B). The sequenator was obtained through a generous grant from the Wallenberg Foundation.

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DEMONSTRATION OF γ -TRACE IN NORMAL AND NEOPLASTIC ENDOCRINE A-CELLS OF THE PANCREATIC ISLETS: AN IMMUNOHISTOCHEMICAL STUDY IN MONKEY, RAT AND MAN

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ABSTRACT

γ -Trace was demonstrated by the immunohistochemical peroxidase/anti-peroxidase technique to be present together with glucagon in the A-cells of pancreas from capuchin monkeys and from rats and in a human islet cell tumor, rich in A-cells, evoking a hyperglucagonaemia (a 'glucagonoma syndrome'). The concentration of γ -trace in plasma was normal in the patient with the glucagon-producing tumor. No cross-reactivity was found when the antiserum against γ -trace was tested with pancreatic glucagon, or when the antiserum against pancreatic glucagon was tested with γ -trace. An optimal fixation for tissues investigated for γ -trace was obtained by the diethylpyrocarbonate vapor fixation of freeze-dried tissue.

KEY WORDS γ -trace / pancreatic islet-cells / gastro-entero-pancreatic (GEP) neurohormones / glucagon-producing tumor ('glucagonoma')

γ -Trace is a low-molecular-weight protein with unknown function, found in human cerebrospinal fluid (CSF), plasma, saliva, semen and urine (3, 8). In healthy adults the average concentration of γ -trace in plasma is 1.1 mg/l and in CSF 5.4 mg/l (10). γ -Trace has recently been demonstrated in human brain cortical neurons (9). The sequence of the 37 amino-terminal amino acid residues of γ -trace demonstrates a slight similarity to the sequence of human pancreatic glucagon, indicating that γ -trace might be related to the peptidergic gastro-entero-pancreatic (GEP) neuroendocrine system (9).

The purpose of this study was to further explore the possible interrelationship between γ -

trace and pancreatic glucagon by investigating immunohistochemically the presence of γ -trace in the islets of Langerhans in normal pancreas of monkey and rat and in an endocrine pancreatic tumor of man, rich in glucagon-producing A-cells.

MATERIALS AND METHODS

Reagents

Diethylpyrocarbonate (DEPC, Pyrokohlensäurediäthylester) was obtained from Merck, Darmstadt, Federal Republic of Germany, 3,3'-diaminobenzidine tetrahydrochloride from British Drug Houses, Ltd., Pool, England, and 4-chloro-1-naphtol from Sigma, St. Louis, Mo., U.S.A. Paraplast was from Scherwood Medical Industries, St. Louis, Mo., U.S.A., and 'white UHU coll glue' from Ligner and Fischer GmbH, BD-7580 Bühl (Baden), Federal Republic of

Abbreviations: CSF, cerebrospinal fluid; DEPC, diethylpyrocarbonate; (h)PP, (human) pancreatic polypeptide; PAP, soluble complexes of horseradish peroxidase / rabbit anti-horseradish peroxidase

Germany. Porcine glucagon and insulin (Actrapid®) were purchased from Novo Industri A/S, Bagsvaerd, Denmark. Human prealbumin was a gift from Dr P. Felding, Department of Clinical Chemistry, Malmö General Hospital, Sweden.

Antisera

A rabbit antiserum against human γ -trace was used in the first step of the peroxidase/antiperoxidase (PAP) technique (13). Its production and specificity have been reported (9). The precipitating titer according to Becker (1) was 0.19 mg γ -trace/ml antiserum. Rabbit antiserum, specific for porcine pancreatic glucagon, without cross-reactivity to glicentin (K 7810), and guinea-pig antiserum against porcine insulin (M 7708) were supplied by Dr J. Thorell, Department of Clinical Chemistry, Malmö General Hospital, Sweden. Antisera raised against somatostatin (R37) and human pancreatic polypeptide (hPP) (734-3), were both gifts from Dr J. F. Rehfeld, Institute of Medical Biochemistry, University of Aarhus, Denmark. Porcine antiserum against rabbit IgG, rabbit antiserum against guinea-pig immunoglobulins and soluble complexes of horseradish peroxidase/rabbit anti-horseradish peroxidase (PAP) were all obtained from DAKO Immunoglobulins, Ltd., Copenhagen, Denmark. Soluble complexes of horseradish peroxidase/guinea-pig anti-horseradish peroxidase were purchased from Polysciences, Inc., Warrington, Pa., U.S.A.

Quantitation

The concentration of γ -trace in plasma from the patient with the glucagon-producing tumor and from one monkey was determined by enzyme amplified single radial immunodiffusion, using the antibody against human γ -trace in the assay and human CSF as γ -trace standard (8). Glucagon in plasma was quantitated by radioimmunoassay.

Biological Tissues and Fluids

Pancreatic tissue was excised from two two-year-old adolescent male capuchin monkeys, *Cebus apella*, 2.6 and 1.7 kg by weight, anesthetized by intramuscular injections of methohexital sodium and from two adult 250 g Sprague-Dawley rats of either sex, anesthetized by inhalation of diethyl ether. The specimens were from the PP-poor regions of the rat pancreas. In addition,

biopsy specimens were obtained at operation from an islet-cell tumor in the tail of the pancreas of a 61-year-old man suffering from diabetes, diarrhoea, stomatitis and a necrolytic migratory erythema, compatible with the clinical picture of the so-called glucagonoma syndrome (11). At operation liver metastases were observed (but no biopsies were taken). Histopathological examination of the excised pancreatic tumor, measuring 25 $\times$ 15 mm, showed a typical endocrine neoplasm. In an immunohistochemical investigation of the tumor cells by the indirect immunofluorescence procedure glucagon immunoreactivity was demonstrated in almost all the tumor cells; only solitary cells showing somatostatin, hPP and neurotensin immunoreactivity. The pre-operative concentration of glucagon in plasma was 5,700 ng/l. Post-operatively (with remaining metastases in the liver and with slight remnant symptoms), the concentration of glucagon decreased to 3,350 ng/l. The concentration of creatinine in plasma was 64 μ M, indicating a normal glomerular function.

Fixation

The specimens were quickly frozen in a propane-butane mixture to the temperature of liquid nitrogen and then freeze-dried and vapor-fixed in DEPC (9). The fixed specimens were vacuum-embedded in paraffin or paraplast and cut in 6 or 2 μ m thick sections which were mounted on slides with 0.15% (w/v) 'white UHU coll glue' in water and air dried.

In order to try some other fixation methods, some specimens were fixed for 24 hr in Bouin's fluid (6), washed in 70% (v/v) ethanol and embedded in paraffin, and others were fixed for 24 hr in 4% (v/v) paraformaldehyde (10% formalin) in 0.1 M phosphate buffer, pH 7.2, at 4°C, washed for 24 hr in 5% (w/v) sucrose in Tyrode solution (7), frozen and cut on a cryostat microtome.

Immunohistochemical Staining

The Sternberger PAP technique was used, but the results of pilot studies indicated that the washing steps should not be allowed to exceed 30 sec. In most experiments the antiserum against γ -trace was highly diluted (1:2,400) when used in the immunostaining procedure, to minimize the interference from the possible presence of unwanted antibodies raised against antigen contaminants.

When two antigens were localized in the same

section with the dual immunostaining technique, the immunohistochemical reaction was carried out in two sequences (13). In the first sequence antiserum against the first antigen was followed by anti-immunoglobulin, PAP, hydrogen peroxide and diaminobenzidine tetrahydrochloride to yield a brown reaction product. In the second sequence, antiserum against the second antigen was again followed by anti-immunoglobulin and PAP, but then by hydrogen peroxide and 4-chloro-1-naphthol to yield a blue reaction product. When the effect of different fixation methods were tested, the indirect immunofluorescence technique was also used (4).

Immunohistochemical Control Procedures

The specific antibodies against human γ -trace were removed from the antiserum by affinity chromatography (9). The antiserum against γ -trace and those against porcine pancreatic glucagon and insulin were all incubated overnight with human γ -trace, porcine pancreatic glucagon and insulin and with human prealbumin in molar amounts corresponding to 20 and 200 times the precipitating titer of γ -trace of the antiserum against γ -trace.

RESULTS

Influence of Fixation

The optimal immunostaining of γ -trace in pancreatic islets was achieved when the freeze-dried, DEPC-fixed and paraffin-embedded tissue was examined by the PAP technique. The indirect immunofluorescence technique could also be used to detect γ -trace. Usable immunofluorescence results were obtained when the specimens were fixed in cold buffered paraformaldehyde, frozen and cut on a cryostat microtome. This fixation, however, did not give sufficient preservation of structure for the PAP technique. After fixation in Bouin's fluid and embedding in paraffin no immunostaining of γ -trace was attained.

Conventional Immunohistochemical Procedure

In sections of pancreas from the two capuchin monkeys there was a distinct PAP immunostaining of γ -trace in the cytoplasm of solitary cells, scattered in the islets of Langerhans (Fig. 1), when the antiserum against γ -trace or the specific antibodies against γ -trace were used. No staining of exocrine pancreatic tissue occurred. When serum from a non-immunized rabbit was used in the first step, no immunostaining was

obtained. When pancreas from rat was immunostained, the γ -trace-containing cells (Fig. 2) and the A-cells containing glucagon were distributed in the same peripheral fashion in the islets. In the PP-poor region of the rat pancreas the cells containing PP-like immunoreactivity, and, to some extent, those containing somatostatin also showed peripheral distribution but their number was smaller. The numerous insulin-containing cells were localized throughout the islets. In sections from the human glucagon-producing tumor there was an intense immunostaining of γ -trace in most of the parenchymal cells (Fig. 3, A and B) as well as of glucagon. The staining was most prominent in those parts of the cytoplasm of the neoplastic A-cells, situated towards the thin strands of connective tissue stroma.

Semi-thin Consecutive Sections

Monkey pancreatic tissue was cut in 2 μ m thick consecutive sections and immunostained by antisera against γ -trace or glucagon. The immunoreactive cells in three islets of Langerhans found in adjacent sections were photographed, drawn and identified. γ -Trace was demonstrated in 100 out of 113 glucagon-immunoreactive cells (88%). Glucagon was observed in 93 out of 98 γ -trace storing cells (95%). When 2 μ m thick adjacent sections from rat pancreas were investigated, the γ -trace-containing cells were found to be identical with those containing glucagon.

Dual Immunostaining of the Same Sections

When sections from monkey pancreas and from the human glucagon-producing tumor were immunostained by anti- γ -trace and anti-glucagon in two sequences, and when the antiserum used in the first step of the first sequence was progressively diluted in serial sections, the color of the immunostained islet-cells turned from brown to mixed brown-blue and finally to exclusively blue (Table 1), indicating that γ -trace and glucagon were present in the same cells. When the antiserum of the first step of the first sequence was omitted, only blue staining was obtained, and when the corresponding antiserum of the second sequence was omitted, only brown color was seen. When anti- γ -trace and anti-insulin were used in the two sequences, different islet-cells stained brown or blue, indicating that γ -trace and insulin were localized in separate endocrine cells.



Fig. 1 Medium-power photomicrograph, showing PAP immunostaining of γ -trace in a pancreatic islet from a monkey. The γ -trace immunoreactive parenchymal cells have the same distribution as the glucagon-producing A-cells. $\times 640$



Fig. 2 Immunohistochemical localization of γ -trace in the peripheral islet-cells in rat pancreas. By the adjacent sectioning technique it could be shown that the γ -trace-containing cells were identical with the glucagon-producing A-cells. $\times 512$



Fig. 3 PAP immunostaining of γ -trace in a glucagon-producing islet-cell tumor of man. γ -Trace is present in those parts of the cytoplasm of the neoplastic A-cells, situated close to the thin strands of connective tissue stroma. A: $\times 250$; B: $\times 512$

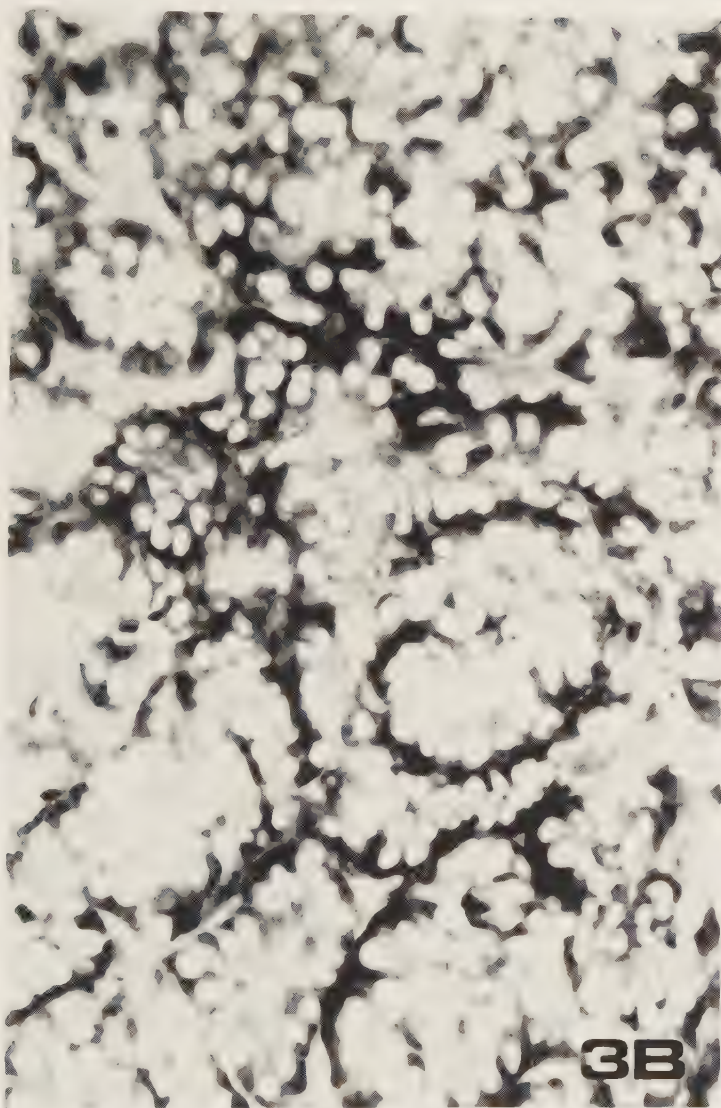


Fig. 3

Antiserum Absorption

The immunohistochemical reactivity of the antiserum against γ -trace disappeared after affinity chromatography, using solid phase γ -trace or after overnight incubation with γ -trace.

There was no alteration in immunoreactivity of this antiserum after incubation with glucagon, insulin, or prealbumin. The antiserum against glucagon was non-reactive after incubation with glucagon, but still reacted when incubated with γ -trace, insulin or prealbumin. The activity of

Table 1 Dual Immunostaining of γ -Trace and Glucagon in the Same Sections of Pancreatic Tissue from Monkey

Dilution of the first sequence	Dilution of the second sequence	Staining of islet-cells
Anti- γ -trace	Anti-glucagon	
1: 600	antiserum omitted	brown
1: 1,800	1: 300	brown
1: 5,400	1: 300	brown and blue
1: 16,200	1: 300	blue
antiserum omitted	1: 300	blue
Anti-glucagon	Anti- γ -trace	
1: 16,200	1: 300	brown
1: 48,600	1: 300	blue
Dual immunostaining in the human glucagon-producing tumor		
Anti- γ -trace	Anti-glucagon	
1: 600	antiserum omitted	brown
1: 1,800	1: 300	brown
1: 16,200	1: 300	blue
antiserum omitted	1: 300	blue
Anti-glucagon	Anti- γ -trace	
1: 1,800	1: 300	brown
1: 16,200	1: 300	brown and blue
1: 48,600	1: 300	blue

the antiserum against insulin disappeared after incubation with insulin, but not after incubation with γ -trace or glucagon.

Endpoints of the Dilution of the Antisera

When tested on normal monkey pancreas and on the human glucagon-producing tumor, the immunoreactive endpoint of serial dilutions of the antiserum against γ -trace was 1: 2,400, but some immunostained islet-cells were still visible when the dilution was 1: 9,600. The solutions of the specific antibodies against γ -trace, recovered from the affinity column at pH 3.5 and 2.2 contained 2.6 g IgG/l and 1.9 g IgG/l, respectively, when measured spectrophotometrically ($A_{280}^{1\%} = 14.3$). The immunoreactive endpoints of the dilutions of these antibodies were of the same magnitude as that of the unabsorbed antiserum.

Concentration of γ -Trace

In the patient with the 'glucagonoma syndrome' the pre-operative value of γ -trace was 1.4 mg/l plasma. After the operation, the concentration of γ -trace was 1.3 mg/l. In one of the monkeys the concentration of γ -trace in plasma was 0.6 mg/l when measured with the assay for human γ -trace.

DISCUSSION

The ideal fixative for immunohistochemical studies differs for various antigens and must, therefore, be tested (2, 12, 14). Even minor variations in the techniques used may influence the results. The fixative should prevent diffusion of the antigen during the procedures of incubation and washing but not destroy its antigenic determinants. It is also essential that the light-microscopical structure of the tissue is preserved. Of the three methods described here, the DEPC-fixation of freeze-dried specimens should obviously be preferred when γ -trace has to be detected immunohistochemically.

As mentioned in the introduction, a slight similarity in amino acid sequence between γ -trace and human pancreatic glucagon has been demonstrated, but the immunostaining with the anti- γ -trace serum was unaltered after absorption in great molar excess with glucagon and no decrease of the immunoreactivity of the anti-glucagon serum was seen after incubation with γ -trace, indicating that the immunostaining was not attributed to a cross-reaction of the antisera.

No cross-reactivity has been found to occur in the sequential dual immunostaining for paired antigens although the same linking antibodies and the same PAP are used in each of the two reaction sequences (13). It is suggested that the

catalytic site of peroxidase and the antigenic determinants of the linking antibodies in the first sequence are blocked by the polymeric product of the diaminobenzidine oxidation.

In healthy adults the average concentration of γ -trace (100 nM) in plasma is more than 3,000 times that of glucagon (30 pM). The increased concentration of glucagon but the normal level of γ -trace in plasma in the man suffering from the glucagon-producing tumor suggests a predominating non-pancreatic origin of the circulating γ -trace. The antiserum against human γ -trace could be used for immunohistochemical investigation of tissues from monkey and rat and for quantitation of monkey γ -trace in plasma, indicating that it contained some antibody populations reacting with γ -trace from these species. The calculated value of the concentration of monkey γ -trace in plasma (0.6 mg/l) was of the same order of magnitude as the value of human γ -trace in plasma.

γ -Trace has been demonstrated in human brain cortical neurons (9). The results of the present study show that γ -trace is also present in the A-cells of the pancreatic islets in normal monkey and rat and in a human glucagon-producing tumor. In addition to the four established islet-cell hormones insulin, glucagon, somatostatin and PP, the plasma protein prealbumin (5) has recently been demonstrated in pancreatic islet-cells. It is not known whether γ -trace and prealbumin are synthesized by the islet-cells or taken up from plasma.

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V.

Demonstration of γ -trace in normal endocrine cells of the adrenal
medulla and in pheochromocytoma.

An immunohistochemical study in monkey, dog and man.

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Running title: γ -Trace occurs in adrenal medulla

Key words: γ -Trace / adrenal medulla / pheochromocytoma /
peptidergic gastro-entero-pancreatic (GEP)
neuroendocrine system

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Abstract. γ -Trace was demonstrated by the immunohistochemical peroxidase/anti-peroxidase technique to occur in normal chromaffin cells of the adrenal medulla from African green monkey, capuchin monkey and beagle dog and in the cells of a norepinephrine-producing human pheochromocytoma. The presence of γ -trace in the endocrine epithelial cells of the adrenal medulla suggests that γ -trace might be related to the neuroendocrine system.

<u>Abbreviations:</u>	Clq,	the first subcomponent of complement
	CSF,	cerebrospinal fluid
	DEPC,	diethylpyrocarbonate
	EDTA,	ethylenediaminetetra-acetic acid = Äthylendinitrilotetraessigsäure
	GEP,	gastro-entero-pancreatic
	PAP,	soluble complexis of horseradish peroxidase/ rabbit anti-horseradish peroxidase

γ -Trace is a low-molecular weight protein with unknown function, found in human cerebrospinal fluid (CSF), plasma, saliva, semen and urine (Colle et al., 1976; Löfberg & Grubb, 1979). In healthy adults the average concentration of γ -trace in plasma is 1.1 mg/l and in CSF 5.4 mg/l (Löfberg et al., 1980). γ -Trace has recently been demonstrated in human brain cortical neurons (Löfberg et al., 1981a), in A-cells of pancreatic islets and in a glucagon-producing islet cell tumor (Löfberg et al., 1981b).

The purpose of this study was to explore the interrelationship between γ -trace and the neuroendocrine system by investigating immunohistochemically the presence of γ -trace in normal adrenal glands from monkey and dog and in a human pheochromocytoma.

Materials and Methods

Reagents. Diethylpyrocarbonate (DEPC, Pyrokohlensäurediäthylester) was obtained from Merck, Darmstadt, Federal Republic of Germany, 3,3'-diaminobenzidine tetrahydrochloride from British Drug Houses Ltd, Pool, England and paraplast from Scherwood Medical Industries, St. Louis, MO., USA. "White UHU coll glue" was from Ligner and Fischer GmbH, BD-7580 Bühl (Baden), Federal Republic of Germany and Heparin-Sepharose CL-6B from Pharmacia Fine Chemicals, Uppsala, Sweden.

Antisera. A rabbit antiserum against human γ -trace was used in the first step of the peroxidase/anti-peroxidase (PAP) technique. Its production and specificity have previously been reported (Löfberg & Grubb, 1979). The precipitating titer (Becker, 1969) was 0.19 mg γ -trace/ml antiserum.

Porcine antiserum against rabbit IgG, fluorescein labeled porcine antibodies against rabbit IgG and soluble complexes of horseradish peroxidase/rabbit anti-horseradish peroxidase were all purchased from Dako immunoglobulins Ltd, Copenhagen, Denmark.

Biological tissues. The adrenal glands were excised from a 13-year-old adult male African green monkey, Cercopithecus aethiops, 5.9 kg by weight, a two-year-old adolescent male capuchin monkey, Cebus apella, 2.6 kg by weight and a two-year-old male beagle dog, 14.4 kg by weight, anesthetized by intramuscular injections of methohexital sodium. In addition, biopsy specimens were obtained at operation from an adrenal tumor of a 30-year-old woman suffering from paroxysmal hypertension accompanied by headache, tachycardia, tremor, anxiety and dizziness, compatible with the clinical picture of a pheochromocytoma. Pre-operatively, the patient was treated for 2.5 months with the α -receptor blocking drug, phenoxybenzamine hydrochloride, administered orally in a dose of 10 mg twice daily and with propranolol chloride in a dose of 20 mg four times daily. At operation, an adrenal tumor, measuring 35 x 35 x 30 mm, adjacent to the right kidney was excised. Histopathological examination revealed a picture consistent with a pheochromocytoma but without invasion in vessels or capsule. Chromaffin staining indicated that the tumor contained catecholamines. When all drugs were omitted, the pre-operative values of 3-methoxy-4-hydroxy mandelic acid in urine was 66-92 μ moles/24 hrs (normal value <35), methoxy-catecholamines in urine 9-19 μ moles/24 hrs (normal value <6), norepinephrine in urine 345-793 nmoles/24 hrs (normal value <300), epinephrine in urine 32-51 nmoles/24 hrs (normal value <60) and aldosterone in urine 73-153 nmoles/24 hrs (normal value <40). Post-operatively, the values were reverted to normal.

Fixation. The specimens were quickly frozen at operation in a propane-butane mixture to the temperature of liquid nitrogen and then freeze-dried and vapor-fixed in DEPC (Pearse & Polak, 1975). The fixed specimens were vacuum-embedded in paraffin or paraplast and cut in 4 or 2 μ m thick sections, which were mounted on slides, using 0.15% (w/v) "white UHU coll glue", diluted in water and then air dried at 37°C.

Immunohistochemical staining. The Sternberger PAP technique (Sternberger & Joseph, 1979) was used; however, the washing steps did not exceed 30 sec. The indirect immunofluorescence technique (Coons et al., 1955) was also used for comparative purposes.

Immunohistochemical control procedures. The specific antibodies against human γ -trace were removed from the antiserum by affinity chromatography (Löfberg et al., 1981a). The antiserum against γ -trace was also incubated over night with human γ -trace in an amount corresponding to 20 times the precipitating titer of the antiserum. Complement subcomponent Clq was removed from the antiserum by affinity chromatography on a column of Heparin-Sepharose CL-6B, equilibrated in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl and 0.02 M EDTA. The Clq-free antiserum against γ -trace was then concentrated to the original volume (McKay, 1981).

Results

Immunohistochemical staining. In sections of the adrenal medulla from monkey (Fig. 1) and dog there was a distinct immunostaining of γ -trace in the cytoplasm of all epithelial cells, when the antiserum against γ -trace, the specific antibodies against γ -trace or the Clq-depleted antiserum against γ -trace were used. No staining of the adrenal cortex occurred. When serum from a non-immunized rabbit was used in the first step of the assay, no immunostaining was obtained. The epithelial cells of the adrenal medulla were identified by their argyrophil reaction when the sections were stained with silver nitrate (Grimelius, 1968). In sections of the human pheochromocytoma there was immunoreactivity of γ -trace in the cytoplasm of all tumor cells, constituting solid nests of polygonal cells and groups of spindle-shaped cells, arranged in whorles and streaks. γ -Trace was also demonstrated in the adrenal medulla and in the pheochromocytoma, when the indirect immunofluorescence technique was used.

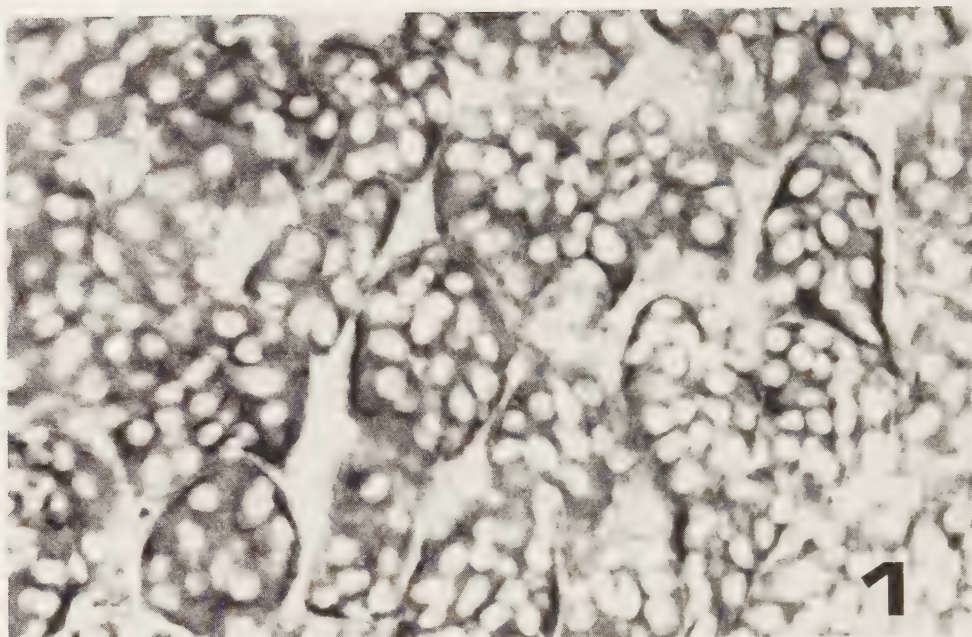


Fig 1 Medium-power photomicrograph, showing PAP immunostaining of γ -trace (dark color) in the cytoplasm of the normal epithelial cells of the adrenal medulla in monkey.

X 640

Antiserum absorption. The immunohistochemical reactivity of the antiserum against γ -trace disappeared after affinity chromatography, using solid phase γ -trace, or after over night incubation with γ -trace.

Endpoints of the dilution of the antisera. When tested on normal monkey adrenal medulla and on human pheochromocytoma, the immunoreactive endpoint of serial dilutions of the antiserum against γ -trace was 1:2,400. The solutions of the specific antibodies against γ -trace, recovered from the affinity column at pH 3.5 and 2.2 contained 2.6 g (IgG/l and 1.9 g IgG/l, respectively, when measured spectrophotometrically ($A_{280}^{\%} = 14.3$). The immunoreactive endpoints of the dilutions of these antibodies were of the same magnitude as that of the unabsorbed antiserum against γ -trace.

Discussion

With proper handling and fixation of biopsy specimens the immunoreactivity of γ -trace was demonstrable. By using high dilution of the antiserum against γ -trace, an incubation buffer with high ionic strength (0.5 M NaCl) and an antiserum containing no Clq (Buffa et al., 1979) the non-specific staining was minimized. No evidence of undesired specific staining of other antigens in the adrenal medulla was found following absorption of the antiserum, using solid phase γ -trace. When the eluted specific antibodies against γ -trace were used in the first step of the assay, the immunostaining was identical to that of the unabsorbed antiserum.

The adrenal medulla is suggested to contain biologically active peptides in addition to norepinephrine and epinephrine (Pearse, 1976) and constitutes a part of the amine precursor uptake and decarboxylation (APUD) system (Pearse, 1969), which now also is called the peptidergic gastro-entero-pancreatic (GEP) neuroendocrine system. Recently, met-enkephalin- and somatostatin-like immunoreactivities (Lundberg et al., 1979), as well as met-enkephalin containing polypeptides (Lewis et al., 1980), have been demonstrated to occur in human adrenal chromaffin cells and in tumors of this cell type. Vasoactive intestinal poly-

peptide (VIP) has also been found in such tumors (Sullivan et al., 1978).

The discovery of γ -trace being present in the medullary epithelial cells of the adrenals, and the previous demonstration of γ -trace in normal and neoplastic glucagon-producing A-cells of pancreas and in neurons in the grey matter of the human brain suggest that γ -trace is closely related to the peptidergic GEP neuroendocrine system and occurs in the cytoplasm of some of its secretory cells.

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VI.

Human γ -trace, a basic microprotein:

Its amino acid sequence and presence in the adenohypophysis

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ABSTRACT The amino acid sequence of human γ -trace, a basic microprotein without known function, was determined by automated Edman degradations of the carboxymethylated polypeptide chain and fragments obtained by cyanogen bromide treatment and tryptic digestion after blocking of lysine residues. The single polypeptide chain contained 120 residues (Fig. 1) and the calculated M_r was 13,260. A proline residue at position 3 was partly hydroxylated. The presence of γ -trace in a significant proportion of the cells in the anterior lobe of simian and human pituitary glands was demonstrated by immunohistochemical procedures using a rabbit antiserum against human γ -trace. The tissue localization and amino acid sequence of γ -trace indicated that this protein is connected with the peptidergic gastro-entero-pancreatic neuroendocrine system.

Human γ -trace is an alkaline, low molecular weight protein first described in 1961 as a constituent of normal cerebrospinal fluid and of urine from patients with renal failure (1 - 3). The biological role of γ -trace is unknown, although the protein has been demonstrated to occur in low concentration not only in cerebrospinal fluid and urine but also in plasma, saliva and semen (4 - 6). A sensitive enzyme immunoassay for measurement of γ -trace was recently described (7). It allowed quantitation of the protein in all human fluids investigated and demonstrated that in healthy adults the concentration of γ -trace in cerebrospinal fluid is 5.5 times that in plasma. Recent immunohistochemical studies of γ -trace have shown it to be present in some human brain cortical neurons, in adrenal medulla, and in the A-cells of the pancreatic islets (8-10). Some amino acid sequence similarities have also been found between human glucagon and the amino-terminal part of γ -trace (8). The question whether γ -trace is related to the peptidergic gastro-entero-pancreatic neuroendocrine system has therefore arisen. This report describes the complete amino acid sequence of γ -trace and presents immunohistochemical evidence of the presence of the protein in a large number of cells in the pituitary gland. Experimental details of the work will be published separately.

MATERIALS AND METHODS

Isolation of γ -trace. Urine containing about 20 mg γ -trace per liter from patients with renal failure was collected in bottles containing the protease inhibitor benzamidinium chloride and the antimicrobial agent sodium azide. γ -Trace was isolated from the pooled urine as described previously (8). Its purity was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (11) and agarose gel electrophoresis (12).

Structural determination. Isolated γ -trace was reduced and $\left[^{14}\text{C}\right]$ carboxymethylated in 6 M guanidinium chloride. The carboxymethylated protein was then fragmented by cyanogen bromide treatment (13) or by tryptic digestion after reversible blocking of amino-groups by citraconylation (14). One of the cyanogen bromide fragments (number 3) was further digested with protease from Staphylococcus aureus V 8. On each occasion the fragments were separated by gel chromatography on a column of Sephadex G-50 superfine (1.5 x 190 cm) in 30 % (v/v) acetic acid. The absorbance at 280 nm, the radioactivity (^{14}C), and the ninhydrin-reactivity of the column fractions were measured. All cyanogen bromide fragments and most of the tryptic peptides were pure after the gel filtration, but some tryptic peptides had to be further purified by preparative high voltage paper electrophoresis (15).

Amino acid analysis was performed by standard methods (16) after hydrolysis in 6 M HCl. Automated amino acid sequence analysis by the method of Edman and Begg (17) was carried out on a Beckman 890 C sequencer using the standard Beckman 1 M Quadrol program. All fragments (50-300 nmol) were run in the presence of polybrene (18). Phenylthiohydantoin amino acids were identified by high performance liquid chromatography on a 30-cm Waters μ -Bondapak C 18 column using stepwise elution with methanol-containing buffers (Waters Ass. Inc., Milford, Ma., Internal communication 03-LS-11-76). This detection system identified the phenylthiohydantoin derivatives of hydroxyproline as two peaks at unique positions in the elution diagram. All phenylthiohydantoin amino acids were also identified by thin layer chromatography (19). Carboxyl-terminal amino acid residues were identified after digestion with carboxypeptidase Y (20).

Immunohistochemical procedures. Pituitary glands were taken at operation from a two-year-old, adolescent, male capuchin monkey, Cebus apella, 2.6 kg by weight, a 13-year-old, adult, male African green monkey,

Cercopithecus aethiops, 5.9 kg by weight and at autopsy, 8 hrs post mortem, from a 75-year-old man. The specimens were snap-frozen and freeze-dried, vapor-fixed in diethylpyrocarbonate (21) and vacuum-embedded in paraplast. Tissue sections, 2 μ m thick, were examined with the Sternberger peroxidase/anti-peroxidase technique (22). A rabbit antiserum against human γ -trace was used in the first step of the assay. Its production and specificity have been reported (8) and the precipitating titer according to Becker was 0.19 mg γ -trace/ml antiserum.

RESULTS

Primary structure of human γ -trace. All cyanogen bromide fragments of γ -trace and all tryptic peptides of the citraconylated protein were isolated and used in the determination of the complete amino acid sequence (Fig. 1). The cyanogen bromide fragment 4 was devoid of homoserine and was therefore identified as the carboxyl-terminal fragment. The amino acid residues at virtually all positions in the polypeptide chain were identified in at least two different fragments. The proline residue at position 3 was found to be hydroxylated in all three investigated preparations of pure γ -trace. Hydroxyproline was identified at position 3, both in the uncleaved polypeptide chain and in the corresponding tryptic and cyanogen bromide fragments thereof. It was in all cases demonstrated both by amino acid analyses and by high performance liquid chromatography of the corresponding phenylthiohydantoin derivatives. The hydroxylation degree was about 50 % but varied slightly in three investigated preparations of γ -trace. The polypeptide chain contained 120 residues and had a calculated molecular weight of 13,260.

When the sequence of γ -trace was aligned with known sequences of

hormones belonging to the gastro-entero-pancreatic neuroendocrine system two similarities were found. Both concerned the 35 residue long amino-terminal part of γ -trace which showed sequence similarities with human corticotropin comprising 8 of its 39 residues and with glucagon comprising 7 of its 29 residues (Fig. 2).

Localization of γ -trace in the pituitary gland. Biopsy specimens containing adenohypophyseal tissue were identified by immunohistochemical techniques using antisera against human somatotropin and human corticotropin. In sections from simian and human adenohypophyses there was immunostaining of γ -trace in the cytoplasm of numerous epithelial cells, when native antiserum against γ -trace, specific antibodies against γ -trace or Clq-depleted native antiserum against γ -trace were used (Fig. 3). No immunostaining was obtained, when serum from a non-immunized rabbit was used. The histochemical immunoreactivity of the antiserum against γ -trace disappeared after passage of the antiserum through a column containing insolubilized γ -trace or after absorption of the antiserum with pure soluble γ -trace. The immunoreactive endpoint of serial dilutions of the antiserum was 1:1,600. There was no decrease in the immunoreactivity against γ -trace of the antiserum after over night incubation with human somatotropin, human prolactin, bovine thyrotropin, porcine corticotropin, synthetic corticotropin $_{1-28}$ and porcine glucagon in molar amounts corresponding to about 20 times the precipitating titer of the antiserum.

DISCUSSION

The complete amino acid sequence of γ -trace presented in this work agrees with two earlier published sequences of the amino-terminal part

of the protein (8, 25). The amino acid composition calculated from the sequence also conforms to the one obtained after acid hydrolysis (Table 1). The amino acid sequence of γ -trace contains three pairs (or triplets) of basic amino acid residues (positions 24-25, 53-54 and 92-94) which might be of considerable interest since it is known that several hypophyseal hormones are produced by proteolytic cleavage of prohormones at such pairs of basic amino acid residues (26).

Recent immunohistochemical studies have demonstrated the presence of γ -trace in chromaffin cells of adrenal medulla, A-cells of pancreatic islets and in a number of brain cortical neurons (8 - 10). The results of this work show that γ -trace also is localized in a large proportion of the cells of the adenohypophysis. These localization studies, taken together, point to a tissue distribution of γ -trace suggesting that it might be a part of the peptidergic gastro-entero-pancreatic neuroendocrine system. A connection between γ -trace and the peptidergic neuroendocrine system is also indicated by the amino acid sequence similarities between human corticotropin, glucagon and γ -trace.

LEGEND TO FIGURE 1

The complete amino acid sequence of human γ -trace and the fragments used in the deduction of the sequence. CNBr: Cyanogen bromide fragments; T: peptides isolated from a tryptic digest of citraconylated γ -trace; CNBr 3 S: peptides obtained after digestion of cyanogen bromide fragment 3 with Staphylococcus aureus V 8 protease;

——> : amino acid residue identified by automated Edman degradation; <—— : amino acid released by carboxypeptidase Y digestion; * : partly hydroxylated residue.

LEGEND TO FIGURE 2

The amino acid sequence of human γ -trace compared to the sequences of human glucagon₂₉ (23) and human corticotropin₃₉ (24). Identical residues of the molecules are shown in boxes.

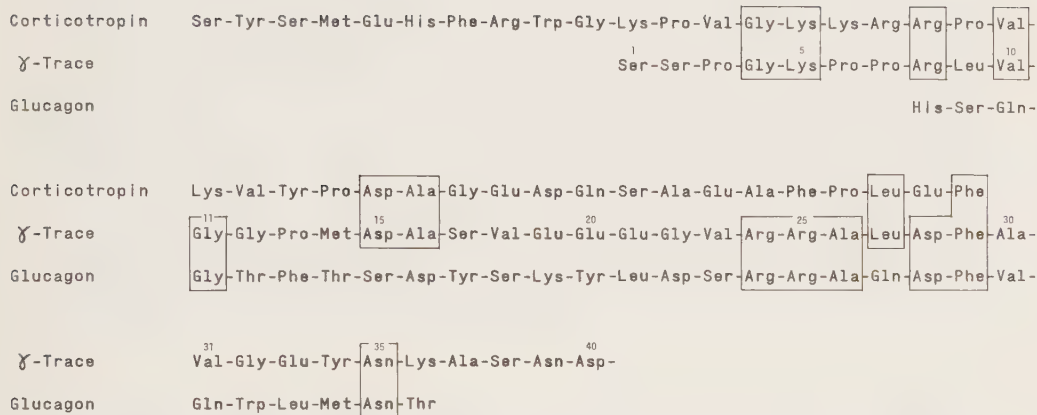


Fig. 2

LEGEND TO FIGURES 3 A AND 3 B

High-power photomicrographs, showing peroxidase/anti-peroxidase immunostaining of γ -trace in the pituitary gland of a 13-year-old, male African green monkey (A) and in a two-year-old, male capuchin monkey (B). γ -Trace (dark color) is present in the cytoplasm of numerous adenohypophyseal cells. Both large cells rich in cytoplasm (A) and small-sized cells (B) contain γ -trace.

A x 822

B x 822

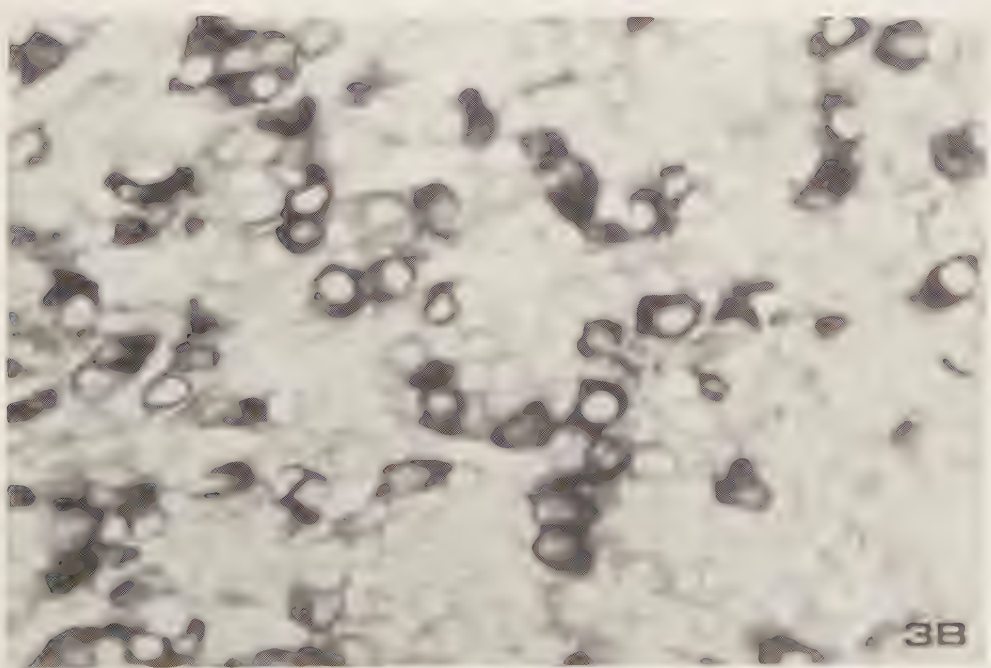
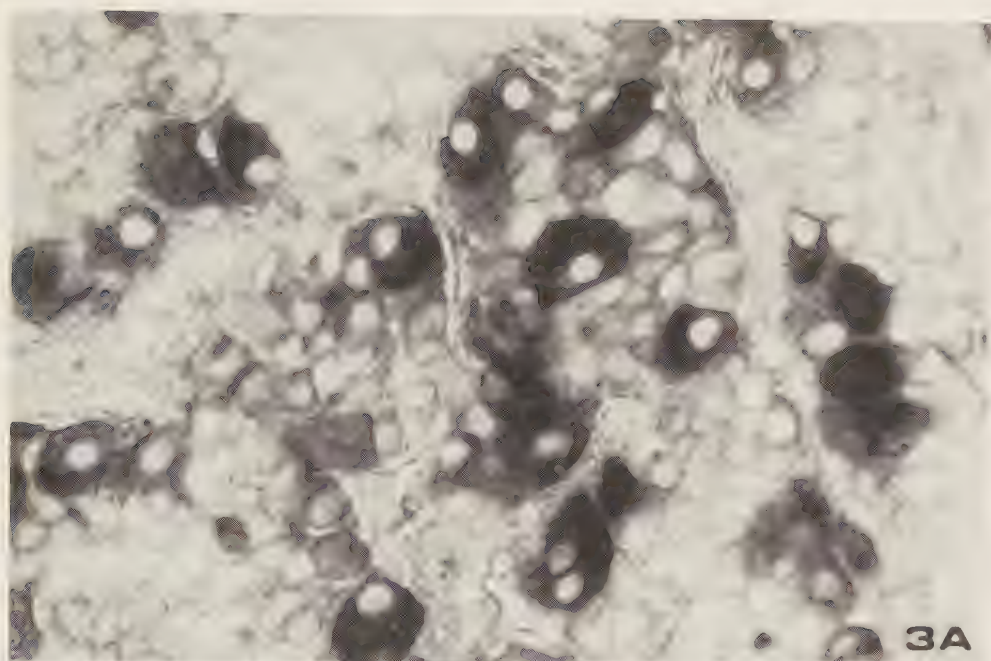


TABLE 1

Amino acid composition of human γ -trace.

Constituent	Composition of acid hydrolysate*	Composition calculated from sequence
Half-cystine	3.7	4
Aspartic acid	12.0	12
Threonine	7.4	7
Serine	8.1	9
Glutamic acid	11.9	12
Proline	8.2	8
Glycine	9.3	8
Alanine	10.0	10
Valine	10.1	10
Methionine	2.4	3
Isoleucine	2.1	2
Leucine	8.2	8
Tyrosine	3.8	4
Phenylalanine	5.1	5
Lysine	6.9	7
Histidine	2.9	3
Arginine	8.0	8

* From Löffberg et al (8). Recalculated to 120 residues.

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